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THESIS

THE NATURE OF TRYPANOSOMATID INFECTIONS OF TABANIDAE IN CENTRAL NEW YORK STATE

WILLIAM LEWIS KRINSKY

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THE NATURE OF TRYPANOSOMATID INFECTIONS OF TABANIDAE IN CENTRAL NEW YORK STATE

A Thesis

Presented to the Faculty of the Graduate School
of Cornell University for the Degree of
Doctor of Philosophy

and

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of Yale University for the Degree of
Doctor of Medicine

by

William Lewis Krinsky

June, 1974

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THE NATURE OF TRYPANOSOMATID INFECTIONS OF TABANIDAE IN CENTRAL NEW YORK STATE

William Lewis Krinsky, M.D., Ph.D.
Cornell University 1974

Horse flies and deer flies of the family Tabanidae were examined for the presence of trypanosomatid parasites. Flies were collected during two consecutive summers from three counties in central New York State using a modified Manitoba canopy trap and an insect net. In addition, tabanids were raised from larvae and were captured in emergence cages. Alimentary tract (including salivary gland) dissections were made. The anatomic distribution and morphology of flagellates were studied. Parity determinations based on ovarian morphology were made. A benzidine test was used for the detection of blood meals.

Only female flies were infected. No freshly-emerged flies were infected. No flagellates were found in the salivary glands. Tabanid intestines harbored amastigote and epimastigote forms. One quarter (27%) of all fly specimens (N=641) representing 69% of the species collected (N=36) were infected. All infected tabanids were gravid, parous and/or blooded.

Trypanosome growth and morphology in N.N.N. (Neopeptone), McCoy's 5a (modified) and CuF_{10} media at 28^{o} and 37^{o} C. were studied. Epimastigotes were frequently found, and trypomastigotes and choanomastigotes rarely found in cultures of tabanid

intestinal flagellates. The intestinal and cultural forms are discussed in relation to the life cycles of Crithidia, Blastocrithidia and Trypanosoma species.

Trypanosoma theileri-like trypanosomes were cultured from the peripheral blood of white-tailed deer and a dairy cow. All epimastigote and trypomastigote forms cultured from tabanids were identical to these ruminant trypanosomes.

BIOGRAPHICAL SKETCH

William Lewis Krinsky was born on 10 January 1947 in Brooklyn, New York. He attended public schools and matriculated at Yale College in 1963. He was an intensive biology major and received a B.A. degree in June, 1967. Following graduation, he attended the Yale University School of Medicine, completing four years of study towards the M.D. degree including a year of graduate study in medical entomology in a Ph.D. program. In 1971, he came to Cornell University to continue his M.D.-Ph.D. program in medical entomology.

He is the co-author of two papers in the fields of mammalian endocrinology and renal pathology. These publications resulted from research done during three summers at the Jackson Laboratory in Bar Harbor, Maine.

He is a member of the Connecticut and New York Entomological Societies, the Entomological Society of America, the American Society of Parasitologists and the American Association of Tropical Medicine and Hygiene.

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NOTE ABOUT PHOTOMICROGRAPHS

All photomicrographs are of giemsa-stained, fixed preparations unless otherwise noted. All living preparations were photographed using phase microscopy; all others, with light microscopy.

The source of all tabanid clone cultures photographed was a Tabanus quinquevittatus collected at Site IV A in 1972.

INTRODUCTION

Flies of the family Tabanidae are known to harbor a diversity of intestinal protozoan flagellates of the family Trypanosomatidae. In 1680, Antony van Leeuwenhoek made the first recorded observations of microorganisms resembling trypanosomes in a horse fly (Dobell, 1958). The specific identity of those flagellated forms is uncertain.

Tabanidae as Hosts and Vectors of Trypanosomatidae

Wallace (1962, 1966), in reviewing the described trypanosomatid parasites of tabanids, grouped most of the parasites into two generic types, Crithidia Léger, 1902 and Blastocrithidia Laird, 1959. A third group of parasites lacked sufficient descriptions to enable generic classification. The majority of described trypanosomatid parasites of horse flies and deer flies are regarded as solely insectan parasites transmitted from fly-to-fly by ingestion of organisms which have come from fly feces. This mode of contaminative transmission of flagellate parasites has been demonstrated several times in various other insect families (e.g., Patton, 1910, Wallace, 1943, Wallace and Clark, 1959; for additional references, see Wallace, 1966), but never in the Tabanidae. No pathological changes associated with trypanosomatid infections of tabanids have ever been recorded.

The genera of the family Trypanosomatidae include soil organisms (Proleptomonas Woodcock), insect parasites (Leptomonas Kent, Herpetomonas Kent, Crithidia Léger, Rhynchoidomonas Patton, Blastocrithidia Laird), plant parasites (Phytomonas Donovan) and vertebrate parasites (Leishmania Ross, Endotrypanum Mesnil and Bremont, Trypanosoma Gruby) (McGhee, 1968; Hoare, 1972). The use of generic prefixes to describe the developmental stages of these diverse but related organisms led to such confusion that Hoare and Wallace (1966) proposed a standard terminology based on the position of the flagellum in each life stage. This terminology, as reviewed by Hoare (1972), and illustrations of the developmental stages are presented in Figure 1.

During their development in an insect vector, trypanosomes of the genera Trypanosoma, Endotrypanum, Leishmania and Phytomonas exhibit forms which are identical to developmental stages of the genera Blastocrithidia, Leptomonas and Herpetomonas (see Figure 2). Therefore, morphological examinations of parasites from insect specimens may be inadequate for distinguishing monogenetic insect parasites from those vertebrate parasites which use insects as vectors or intermediate hosts. Wallace (1962) illustrated this problem by stating that trypanosomatid parasites of tabanids placed in the genus Blastocrithidia Laird, 1959 may actually represent stages in the life-cycle of a Trypanosoma species. The vertebrate Trypanosoma species and other disease agents known to be transmitted by tabanids will be discussed.

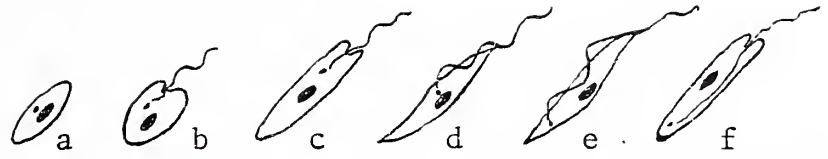


Figure 1. Developmental Stages of Trypanosomatidae (after Hoare, 1972).

a. amastigote - former "leishmanial" stage; rounded or elongate form devoid of external flagellum.

b. choanomastigote - small form usually with antenuclear kinetoplast; flagellum arising from a wide funnel-shaped reservoir and emerging at the anterior end of the body. (Restricted to genus Crithidia.)

c. promastigote - former "leptomonad" stage; elongate form with antenuclear kinetoplast, flagellum arising near it and emerging from anterior end of the body.

d. epimastigote - former "crithidial" stage; elongate form with juxtanuclear kinetoplast; flagellum arising near it and emerging from the side of the body to run along its surface or along a short undulating membrane.

e. trypomastigote - former true "trypanosome" stage; elongate form with postnuclear kinetoplast; flagellum arising near it and emerging from side of body to run along its surface or along an undulating membrane.

f. opisthomastigote - former "trypanosome" or "trypanomorphic" stage; elongate form with post nuclear kinetoplast, flagellum arising near it, then passing through the body and emerging from its anterior end (as in c). (Restricted to genus Herpetomonas.)

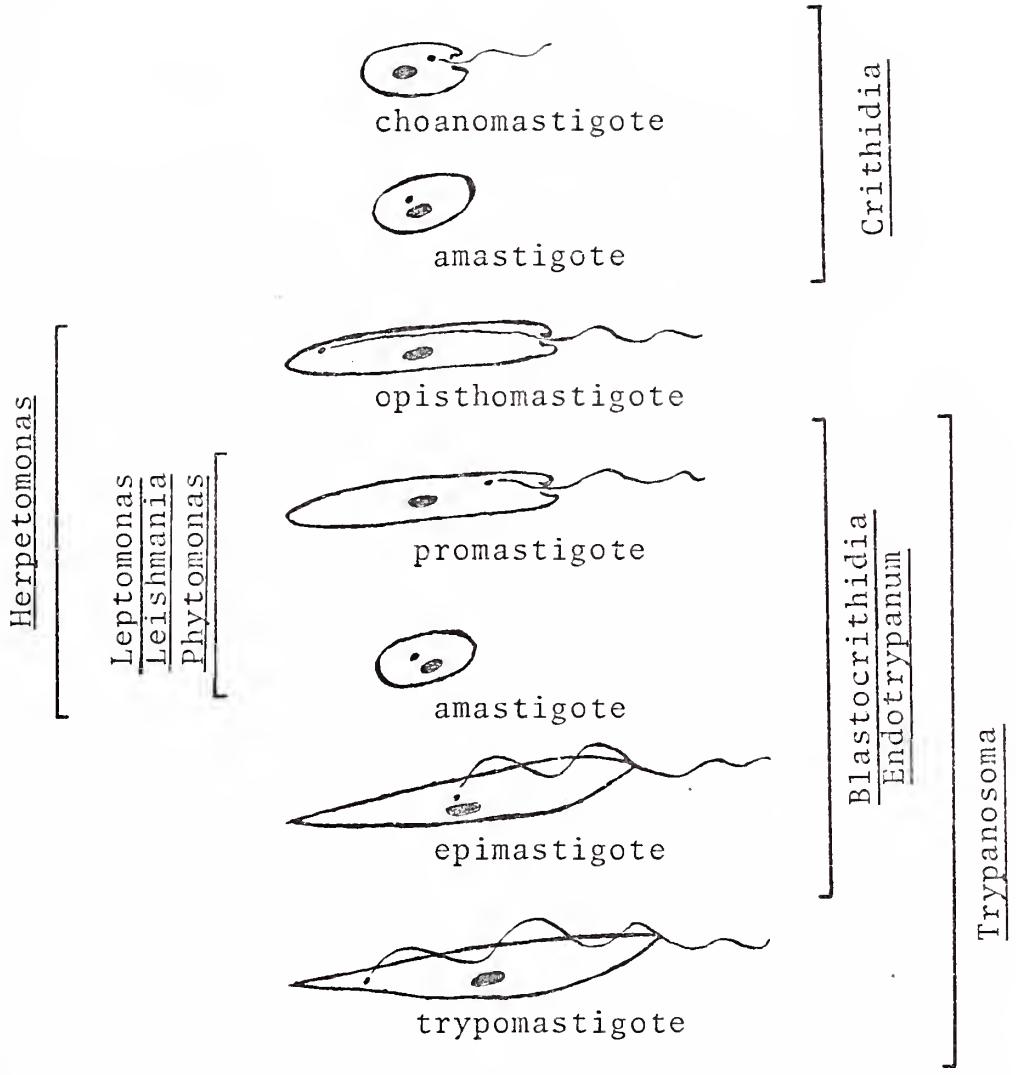


Figure 2. Life Stages Comprising Different Genera of Trypanosomatidae (after Hoare, 1972).

Disease Agents Transmitted by Tabanids

Tabanidae are capable of transmitting a diversity of disease agents. The role of tabanids as vectors of parasites of animals and man has been reviewed by Barretto (1949), LeClercq (1952, 1971), Anthony (1962) and Chvála et al. (1972). A list of disease agents arranged according to their possible modes of transmission by tabanids is given in Table I.

Three encephalitis viruses and the rickettsia Coxiella burneti are tentatively considered capable of being mechanically transmitted by tabanids. These disease agents were isolated from laboratory animals which had been inoculated with pools of ground tabanids. The mere presence of these pathogens in the flies does not constitute proof that the flies act as vectors of these agents. If, indeed, tabanids are vectors, transmission may be mechanical or propagative, as in the case of the yellow fever virus by Aedes aegypti (Whitman, 1937). Issel (1973), in Wisconsin, isolated Jamestown Canyon virus from a white-tailed deer, which was apparently bothered by tabanids and simuliids. This observation, in conjunction with the isolation of the virus from tabanids (DeFoliart et al., 1969), adds support to the theory that tabanids may be vectors of California encephalitis.

Designation of Onchocerca gibsoni as a parasite transmitted by tabanids is also speculative. Cilendo (1923) provided inconclusive evidence for transmission of this parasite by tabanids. Buckley (1938) reviewed the literature regarding

Table I. Disease Agents Transmitted by Tabanidae.

A. Mechanical Transmission[1]

Viruses

California Encephalitis (*)[2]

Jamestown Canyon serotype (DeFoliart *et al.*, 1969)

LaCrosse serotype (Wright *et al.*, 1970)

Equine Infectious Anemia

Hog Cholera (Tidwell *et al.*, 1972)

Rinderpest

Tick-borne Encephalitis (*)

Vesicular Stomatitis

Rickettsia

Coxiella burneti (*) Amanzhulov *et al.*, 1965)

Bacteria

Bacillus anthracis

Bacillus bipolaris balisepticus

Francisella tularensis

Protozoa

Haemosporidans

Anaplasma marginale

[1]Terminology used for modes of transmission is defined by James and Harwood (1969). References to transmission are given after the names of disease agents only in cases where primary references do not appear in the reviews mentioned in the text.

[2](*) indicates that inclusion in the table is based on supporting evidence but transmission has not been demonstrated.

Table I, continued

Trypanosomes

Trypanosoma brucei, *T. congolense*, *T. evansi* (*T. equinum* and *T. hippicum* = New World forms), *T. simiae*, *T. vivax* (Hoare, 1972)

B. Cyclo-Developmental Transmission

Helminths

Elaeophora schneideri (Hibler *et al.*, 1970)

Loa loa

Onchocerca gibsoni (*) (Cilento, 1923)

C. Cyclo-Propagative Transmission

Protozoa

Haemoproteus metchnikovi (*) (DeGiusti *et al.*, 1973)

Trypanosoma spp. (subgenus *Megatrypanum*) (see text)

possible insect transmission of this filarial worm of cattle and provided evidence for its transmission by Culicoides midges.

The recent finding of oöcysts and sporozoites of the chelonian haemoproteid, Haemoproteus metchnikovi in Chrysops callidus (DeGiusti et al., 1973) provides the first definitive evidence of biological transmission of a haemosporidan by a tabanid. The bovid trypanosome T. theileri is the only other protozoan suspected of being biologically transmitted to a vertebrate by tabanids. The evidence for this transmission will be discussed in the context of the history and biology of this trypanosome species and its relatives.

Trypanosoma theileri Laveran 1902

The extensive literature on the isolation, morphology, epidemiology, pathogenicity, taxonomy and life-cycle of T. theileri and related trypanosomes (subgenus Megatrypanum) has been thoroughly reviewed by Wells (1972) and Hoare (1972).

The Parasite

In 1902, Sir Arnold Theiler, working in South Africa (Transvaal), noted a disease in cattle, which had recently been immunized against rinderpest virus. He described the disease as a form of "gall-sickness" (liver derangement) and attributed it to the presence of a large trypanosome. Theiler sent blood films of the trypanosomes to Bruce in London and Laveran in Paris, who independently named the new parasite

Trypanosoma theileri (Laveran 1902a, Bruce, 1902). Laveran's name took priority since it was published five days earlier than Bruce's designation.

Morphology. Theiler (1903) noted that the new trypanosome was similar to "the allied parasites of the tsetse disease of South Africa, the Mal de Caderas of South America, the Surra in India, and la Dourine in Algeria"; however, he distinguished it from these by its large size and polymorphism. Two forms of the parasite were observed in stained preparations of fresh blood. One ("ordinary form") had the "centrosoma" (kinetoplast) at the posterior end of the body and the "rarer form" had it near or on the nucleus. The parasite was 20-70 μ long and 2-6 μ wide. The nucleus was large and spherical, oval, dumbbell-shaped, or, rarely, spindle-shaped. The trypanosome had an undulating membrane bordered by the flagellum and the "protoplasma" contained deeply staining granules. The "rarer form" was broader, often with a larger nucleus, and less compact than the "ordinary form." It exhibited abnormal shapes, e.g., "round, oval, lacerated." Theiler regarded both of these forms as one species since blood containing the "rarer forms" when inoculated into susceptible cattle produced the "ordinary forms." Also, both forms, as well as intermediate forms, were often observed in the same animal. Laveran (1902b) regarded the forms as two species, T. theileri and T. transvaaliense ("rarer form"); however, eventually he accepted Theiler's view (Laveran and Mesnil, 1907). Living specimens of the trypanosomes exhibited both forward movement (flagellum in front) and backwards

movement (flagellum behind).

Theiler (1903) noted that the parasite divided by longitudinal fission. He observed various intermediate stages of division, i.e., cells with two nuclei, one "centrosoma" (kinetoplast) and one flagellum and rounded forms having one or two nuclei associated with two "centrosomes" (kinetoplasts). These peculiar forms could not be easily separated as longitudinally and transversely dividing cells.

Pathogenicity. The severity of the disease ("gall sickness") was often unrelated to the number of trypanosomes observed in an animal's blood and, in some sick animals, the trypanosomes were never found. Theiler attributed these facts to the presence of immunity resulting from previous exposure to the trypanosomes.

Theiler's difficulty in consistently finding the new trypanosome in association with the disease was explained in 1910, when Theiler himself recognized the haemosporidan Anaplasma as the etiologic agent of "gall sickness." Since then, T. theileri has been regarded as a cosmopolitan, non-pathogenic organism (Herbert, 1964). T. theileri has often been found in small numbers in the blood of healthy cattle. It is usually detected in such cases only by culturing the blood (Hoare, 1972).

T. theileri has also been isolated from bovids with a diversity of pathogenic problems including encephalitis (Carpano, 1932), a disease resembling anthrax (Wyssman, 1935), "turning sickness" (Carmichael, 1939), abortion, diarrhea and rumen atony (Levine et al., 1956), depressed milk production and

eosinophilia (Ristic and Trager, 1958), fever and diarrhea (Communicable Disease Center, 1966), penile hematoma (Ewing and Carnahan, 1967) and bovine lymphocytosis (Cross et al., 1968; Hare et al., 1970). T. theileri has also been isolated from an aborted fetus (Dikmans et al., 1957) and a comatose term fetus which died on the day of birth (Lamy and Bouley, 1967). The observations of T. theileri in association with the array of distinct disease entities mentioned above indicate that T. theileri is an opportunistic parasite capable of proliferation in a host debilitated by stressful conditions or intercurrent infection. Past observations of high parasitemias among cattle undergoing rinderpest immunization, which in itself often caused fever (Herbert, 1964), support this view. If T. theileri is an opportunistic parasite, it is not clear whether it acts as a pathogen under conditions which favor its multiplication.

Taxonomy. Hoare (1964), in his revision of the systematics of the mammalian trypanosomes, introduced subgenera and designated Trypanosoma (Megatrypanum) theileri Laveran 1902 as the type-species of the subgenus Megatrypanum.

Trypanosomes resembling T. theileri Laveran 1902 have been observed in domestic and wild ruminants* from all parts of the world. Wells (1972), following the conservative suggestion of Keymer (1967), accepted the designation T. theileri only for

*Parasites have been observed primarily in the blood of Bovidae and also in Camelidae, Tragulidae, and Cervidae (Wells, 1972). (See Section "The Host.")

Theiler's original parasite and listed all other Megatrypanum ruminant species (with the exception of T. melophagium (Flu) Nöller of sheep) as "T. theileri-like" trypanosomes.

Hoare (1972) recognized five distinct species of Trypanosoma (Megatrypanum) from ruminants:

	hosts
T. (M.) theileri Laveran	cattle, antelopes, chevrotains
T. (M.) ingens Bruce, Hamerton, Bateman and Mackie	cattle, antelopes, chevrotains
T. (M.) mazarum mazza Romana and Fiora	brocket deer (Mazama spp.)
T. (M.) melophagium (Flu) Nöller	sheep, camels
T. (M.) theodori Hoare	goats

The Host

The hosts of the Megatrypanum species recognized by Hoare (1972) are listed above. Wells (1972) listed observations of T. theileri-like trypanosomes from Bovidae, i.e., domestic cattle (Bos taurus, Bos indicus), buffalo (Asiatic and African), bison and antelopes as well as Camelidae (Arabian camel), Tragulidae (chevrotains) and Cervidae (brocket deer).

Until recently, with the exception of brocket deer (Mazama spp.) from South America, the Cervidae were not known to harbor T. theileri-like trypanosomes. Kistner and Hanson (1969) cultured trypanosomes from the blood of white-tailed deer (Odocoileus virginianus) in six Southeastern states. These trypanosomes, which were the first ever isolated from white-tailed

deer in the United States, were morphologically similar to the large ruminant Megatrypanum species. D'Alessandro and Wells (1971) isolated T. theileri-like trypanosomes from peripheral blood of Colombian Cervidae (Odocoileus virginianus gymnotis and Mazama guasubira medemi). In 1972, Clark reported isolation of Trypanosoma sp. from mule deer (Odocoileus hemionus) in New Mexico and Colorado. The first observations of trypanosomes from elk (Cervus canadensis) were made in Wyoming and New Mexico in 1972 and 1973 (Davies et al., 1973; Kingston and Morton, 1973).

All of the above trypanosomes isolated from cervid species demand further morphological and biological study to determine their relationships with the T. theileri-like complex of wild and domestic ruminants.

Repeated unsuccessful attempts to infect birds and nonruminants with T. theileri-like organisms from infected cattle (Theiler, 1903; Curasson, 1943; Wells, 1972) indicate that these trypanosomes are host-specific for the sub-order Ruminantia.

The Vector

Theiler (1903) transmitted T. theileri from infected calves to clean calves in two of four attempts, by allowing several starved hippoboscids to feed consecutively on the two groups of animals. The hippoboscids were later identified as two species, Hippobosca rufipes v. Olfers and H. maculata

Leach (Laveran, 1903). Control animals kept in the same stable with the experimental ones failed to become infected. Theiler suggested that other blood-sucking flies, such as tabanids, may also be vectors; however, he did not attempt transmission with tabanids.

In the decade following Theiler's discovery, several investigators in different parts of the world observed cattle trypanosomes similar or identical to T. theileri, for example, in other parts of Africa* (Algeria, Angola, Ethiopia, Malawi, Nigeria, Senegal, Tanzania, Togo, Tunisia, Uganda, Zaire, Zambia), Eurasia (Denmark, England, Finland, France, Germany, Greece, Holland, Portugal, Sweden; China, India, Indonesia, Japan, Philippines, Singapore, U.S.S.R.) and North and South America (U.S.A., Canada; Argentina, Brazil, Uruguay) (Wells, 1972). However, no additional information about the mode of transmission of the parasite was obtained until 1916, when Nöller, in Germany, cultured the intestines of Tabanus glaucopis specimens and observed flagellate protozoans, which he claimed were morphologically identical to culture forms of T. theileri from cattle (Nöller, 1916; Hartmann and Nöller, 1918).

Nöller (1916) and, more recently, Herbert (1964) and Hoare (1972) regarded Theiler's transmission of T. theileri by hippoboscids as mechanical transmission. A discussion of Nöller's findings (1916) is given below.

*Recent (1974) names of African countries are used here.

Nöller dissected the intestinal tracts of 15 female Tabanus glaucopis specimens which he had caught around a horse in Germany. The hindgut of one of the flies was infected with flagellates, which Nöller claimed resembled Crithidia subulata Léger 1904. The name Crithidia subulata was an emendation by Patton (1907) of Herpetomonas subulata Léger. In the reference cited by Nöller, Léger (1904) described Herpetomonas subulata consisting of two forms, i.e., "formes grégariniennes" (now recognized as choanomastigote forms) and "formes monadiennes" (now recognized as epimastigote forms).* Patton's emendation (1907) represented a complete misinterpretation of the original description of the genus Crithidia Léger 1902 (characterized by choanomastigote forms). Patton assumed, without any experimental evidence, that all choanomastigote forms were "young" forms and all slender, spindle-shaped forms were "adult" forms of insect flagellates (Wallace, 1943). Following this reasoning, he made the astonishing statement that the "formes grégariniennes" were early stages of a Crithidia and that the "formes monadiennes" were the adult stages of a Crithidia (Patton, 1907). Thus, Patton decided that a true Crithidia had incorrectly been named Herpetomonas. Using this very unconventional approach to taxonomic principles, Patton implied that a true Crithidia was

*Recently, Wallace (1962) assigned the "formes monadiennes" of Herpetomonas subulata Léger 1904 to their appropriate taxonomic position as Blastocrithidia subulata (Léger, 1904) Wallace, 1962. In addition, Wallace (1962), following the original description of Crithidia Léger, 1902, grouped the "formes grégariniennes" in their rightful position as Crithidia spp.

different from the original description of this genus.

Nöller's description of the flagellates from T. glaucopis is consistent with the illustrations of non-flagellated forms of Herpetomonas subulata Léger 1904 ("formes grégariniennes") and not the "formes monadiennes" which Patton (1907) erroneously regarded as "adult" stages of Crithidia subulata. Nöller's use of the latter name represents one example of many in which the acceptance of Patton's emendation (1907) led to the complete confusion of the taxonomy of the insect flagellates (Laird, 1959).

The taxonomic classification of Nöller's flagellates from T. glaucopis is still open to question. Those parasites were described as "barley corn" forms having the "blepharoplast" (kinetoplast) at the hind end. Nöller (1916) noted their resemblance to the elliptical or oval resting stages of Léger's parasite (i.e., "formes grégariniennes" discussed above). No forms with an undulating membrane or free flagellum were observed. No parasites were found in the midgut; all were described as located below the attachment of the Malpighian tubules. Many of the parasites were attached to the gut wall. Wallace (1962) stated, "In size, shape, location and in being attached to the gut wall the flagellates Nöller described in the insect (T. glaucopis) would now be identified as Crithidia." However, the hind position of the kinetoplast is not consistent with the generic description of Crithidia. In addition, as mentioned earlier, Nöller cultured washings from the infected hindgut in blood (goat) agar bouillon at room temperature and, in three

days, observed typical cattle trypanosomes identical to culture forms of T. theileri (i.e., epimastigote forms). Wallace (1962) accepted the fact that Nöller's cultured organisms were Blastocrithidia or Trypanosoma. He tried to reconcile the apparent discrepancy between the insect forms and the culture forms by introducing the possibility that the fly Nöller cultured had a mixed infection.

In 1925, Nöller isolated three strains of flagellates from Haematopota pluvialis collected near Berlin. When these were cultured on blood agar plates, forms identical to those obtained in 1916 from T. glaucopis were observed. Nöller selected three calves and one steer in which he was unable to demonstrate flagellates by blood culture. The cattle were inoculated with the fly cultures. Two of the three calves (aged 5 days) became infected after single inoculations, as evidenced by the growth of flagellates in blood cultures. The steer (aged 7-9 months) failed to become infected until three separate cultures had been inoculated. One of these cultures (the last) exhibited "barley corn" forms similar to those forms seen in the flies.

Flagellates cultured from the cattle were indistinguishable from culture forms of T. theileri. Wallace (1962) reviewed the details of the inoculation study and accepted the fact that transmission of T. theileri from fly cultures to cattle had been proved. However, he failed to accept the idea that those forms which Nöller described in the fly gut (which Wallace recognized as Crithidia) represented the infective resting forms

of T. theileri. Wallace cited Clark (1959) as evidence that "the insect stage of a trypanosome (i.e., Trypanosoma sp.) can not resemble Crithidia but must resemble Blastocrithidia." Actually Nöller's insect forms (having posterior kinetoplasts) are more consistent with a description of a resting precursor form of Trypanosoma than with the description of Crithidia.

Whether one accepts Nöller's description of the insect stage as that of the vector form of T. theileri or not, the validity of his transmission study may be questioned. Two apparent characteristics of the mammalian forms of T. theileri, i.e., their cryptic behavior during the winter months and their ability to cross the placental barrier, indicate the extreme difficulty of selecting uninfected cattle for experimentation.

Crawley (1912) and others (Wells, 1971b, 1972) had difficulty or found it impossible (Behn, 1912) to isolate trypanosomes from known infected cattle during the winter months. Behn (1912) assumed that the cows lost their infection each winter and were reinfected each spring. This would presume that the trypanosomes overwinter in some stage of the invertebrate vector or, perhaps, in an intermediate host. Present knowledge of other trypanosome species make both of these possibilities very unlikely. Furthermore, there are several examples of trypanosome species which reproduce as amastigote forms in the tissues, e.g., T. cruzi (Hoare, 1972), or occupy cryptic (tissue) foci (T.-W.-Fiennes, 1952; Goodwin, 1970; Seed and Effron, 1973) in the vertebrate host.

Nöller (1925) obtained his control cultures and inoculated

calves during the fall and winter months. Therefore, it is possible that the infections discovered after inoculation were always present but previously undetected. Furthermore, evidence for prenatal transmission of T. theileri (Dikmans et al., 1957; Lundholm et al., 1959; Cummunicable Disease Center, 1966; Lamy and Bouley, 1967; Woo and Limebeer, 1971; Moulton and Krauss, 1972) raises the possibility that Nöller's young calves may have acquired the infections during gestation.

In 1931, Kraneveld reported the results of an extensive series of transmission studies of T. theileri by Tabanidae in the Dutch Indies (Indonesia). Approximately 20% of each of the two tabanid species, Tabanus striatus (3504 females) and T. rubidus (4862 females), dissected harbored flagellates in the intestinal tract. In addition, flagellates were found in four other species (three Tabanus and one Haematopota). The morphological classification of these flagellates is not clear. Apparently, epimastigotes and oval, small forms were observed in the midgut and hindgut (Wells, 1972). Kraneveld, who was aware of Nöller's experiments (1925), was convinced that tabanids were vectors of T. theileri. His experiments were designed specifically to demonstrate the mode of transmission of T. theileri from tabanids to oxen and water buffaloes.

Four kinds of transmission were attempted with wild specimens of each tabanid species (T. striatus and T. rubidus): (1) tabanids were allowed to feed on the bovids; (2) fresh tabanid intestines were mashed in sterile saline and spread over the unbroken membranous portion of the mouths of bovids; (3) fresh

tabanid intestines (prepared as in 2) were placed on the intact vaginal mucosae of bovids; and (4) tabanid feces were spread on the unbroken membranous portions of the mouths of bovids. Large numbers of flies were used and blood samples from bovids (kept in fly-free stables) were cultured for T. theileri several times prior to the experiments. Animals selected for the experiments had consistently negative cultures. Experiments (1), (2) and (3) with T. striatus resulted in transmission, as evidenced by the appearance of positive blood cultures for T. theileri. The results of experiment (4) with T. striatus and all those with T. rubidus failed to demonstrate transmission. Though the wild flies were held in the laboratory for 39 to 99 hours prior to each experiment, the possibility of mechanical transmission in experiment (1) was not ruled out. The failure to demonstrate transmission with fecal matter and the failure of all the experiments with T. rubidus could not be explained. Kraneveld noted that flagellate forms observed in T. striatus and T. rubidus were morphologically identical and, in addition, flagellates from both species, when cultured in blood bouillon and glucose-supplemented agar plates, produced forms identical to T. theileri cultures from native bovids. Kraneveld speculated that the flagellates from T. rubidus were parasites of the flies themselves.

The ability of tabanid intestinal material to infect animals across intact mucous membranes (mouth and vagina) suggested to Kraneveld (1931) that T. theileri might be transmitted by ingestion of the vector or by contamination of the host's

mouth with infective material from the vector. Such a mode of contaminative (stercorarian) transmission had already been demonstrated with Trypanosoma lewisi (Strickland, 1911 and others) and T. melophagium (Hoare, 1923).

Recently, Page (1972) dissected four species of tabanids (614 females) from Colombia. He found 3.4% of the flies infected with intestinal flagellates. Some of the infections,which were comprised of "non-motile heart-shaped forms and ... associated motile forms" Page considered Crithidia species. He speculated that the remaining infections, characterized by spindle-shaped organisms, represented vector stages of Trypanosoma theileri.

Observations of epimastigote flagellates in other haematophagous arthropods, e.g., Stomoxys (Jegen, 1924) and Hyalomma aegyptium (O'Farrell, 1913), led to suggestions that these species may act as vectors of T. theileri; however, no experimental studies have ever been done. Glaser (1922) fed cultures of T. theileri-like trypanosomes to ten Haematobia serrata and twenty Stomoxys calcitrans and noted the survival of the flagellates in the alimentary tracts of these species for up to 48 hours. No transmission studies were attempted.

Thus, Theiler (1903), Nöller (1925) and Kraneveld (1931) provided the only experimental evidence for transmission of T. theileri by insect vectors. Theiler's transmission study, using hippoboscids, is now considered an example of mechanical transmission (as mentioned above). Nöller's experiments failed to definitively identify the insect stage of T. theileri.

Kraneveld was unable to distinguish between tabanid trypanosomatids (from T. striatus) which resulted in T. theileri infection and those (from T. rubidus) which may have been monogenetic insect parasites. Kraneveld's experiments also suggested that T. theileri may be transmitted by the bite of the vector (salivarian transmission).

Blastocrithidia Species in Haematophagous Insects

The genus Blastocrithidia Laird, 1959 was established to describe epimastigote forms* occurring in invertebrates which had been incorrectly called Crithidia by Patton (1907, 1909). Patton, as mentioned earlier, disregarded the original description of the genus Crithidia Léger, 1902 and assumed that the choanomastigote forms described by Léger (1902) must have been "young" forms and that the true Crithidia "adults" must be epimastigote forms (Wallace, 1943).

The confusion in the literature resulting from Patton's emendation was compounded by the common practice of calling the vector epimastigote stages of vertebrate Trypanosoma species "crithidia forms." Wallace (1943) revived the original description of Crithidia as stated by Léger (1902) (i.e., choanomastigote forms) and, in doing so, revealed that the Trypanosoma "crithidia" forms are named after a genus which they do not

*Modern terminology for the morphologic descriptions of flagellate life stages (Hoare, 1972) is used throughout this discussion. (Figure 1.)

resemble. Laird (1959) noted that the epimastigote forms wrongly named Crithidia by Patton (1907, 1909) lacked a generic name. For these monogenetic insect parasites, which never progress beyond the epimastigote stage of development, Laird coined the term Blastocrithidia (lit. = "sprouting" + Crithidia).

Wallace (1966) reviewed the Blastocrithidia species known from insects and ticks. As mentioned earlier, epimastigote forms observed in haematophagous insects may represent Blastocrithidia species or Trypanosoma species; however, without biological (life-cycle) studies, it is impossible to separate this stage of the two genera. The life-cycle of at least one Blastocrithidia species from a haematophagous insect (B. pulicis (Porter, 1911) Wallace, 1966 from Pulex irritans) has been studied in detail in laboratory insects (Porter, 1911), providing evidence that the designation of some epimastigote forms from blood-sucking insects as Blastocrithidia is biologically valid. In addition, Cerisola et al. (1971) provided biological evidence for the existence of Blastocrithidia triatomae in Triatoma infestans (from Argentina), a common vector of Trypanosoma cruzi. However, most of the other Blastocrithidia species from haematophagous insects (including all of those from Tabanidae) (Wallace, 1966) are included in the genus on the basis of morphological descriptions. Any of these species may actually be developmental forms of a vertebrate Trypanosoma species.

Wallace (1966) listed Blastocrithidia species from Chrysops (2 spp.*), Haematopota (2 spp.), Pangonia (1 sp.) and Tabanus

*Species numbers in parentheses refer to fly species.

(5 spp.). Hoare (1972), apparently arbitrarily, listed several of these tabanid Blastocrithidia species as synonyms of Trypanosoma theileri Laveran 1902.

On the basis of Hoare's (1972) slight revision of the description of "epimastigote"*, Crithidia minuta Léger 1903 (partim) from Tabanus tergestinus should be added to Wallace's list (1966) of Blastocrithidia (epimastigote) species. In addition to describing a typical choanomastigote form (Crithidia), Léger (1903) described and illustrated an elongate, tapering flagellate. The flagellum of this form arose near the nucleus and ran along the edge of the cell before it became a short free flagellum anteriorly. Léger observed (in the fly intestine) radial masses of these forms, comprised of individual flagellates attached to each other by their anterior ends.

An additional observation of tabanid flagellates (not mentioned by Wallace, 1962, 1966) was reported by Nieschulz (1931). He found flagellates in 20% of three species of flies (Tabanus rubidus, T. striatus, T. rufiventris) dissected in Java. Nieschulz described them as identical to Crithidia subulata Léger. Nieschulz was apparently referring to Herpetomonas subulata Léger "formes grégariniennes" in his use of Patton's inappropriate emendation (1907). Nieschulz presumed, as did Nöller

*Hoare and Wallace (1966) coined the term "epimastigote" as a form with the flagellum arising near the nucleus and "emerging from the side of the body to run along a short undulating membrane." Hoare (1972) described "epimastigote" as a form with the flagellum arising near the nucleus and "emerging from the side of the body to run along its surface or along a short undulating membrane" (underlining added).

(1916) in Europe, that these forms represented developmental stages of Trypanosoma theileri.

The original observations of all the Blastocrithidia species now known from tabanids were made between 1904 and 1959, with all but two before 1925. None of the observations of tabanid Blastocrithidia were made in the United States. Only four investigators have ever recorded trypanosomatids of any genus from North American tabanid species. Two of these (Clark, 1972; Davies et al., 1973) from the Western United States failed to give any description of the flagellates found. Packchanian (1957), in Texas, observed two "crithidial" species, one recorded from "Chrysops sp." and the other from "Tabana atratus." No detailed morphological descriptions of these flagellates were given. However, the flagellate from "Tabana" was cultured and Packchanian claimed that the cultured forms as well as the initial smear from the insect gut were indistinguishable from the cultured forms of T. theileri from cattle. Wallace (1962) isolated and named Crithidia rileyi, a choanomastigote parasite of Tabanus epistates in Minnesota.

Goals of the Present Study

Three factors stimulated the present research: (1) the present state of confusion concerning the taxonomy of tabanid intestinal epimastigote flagellates, (2) the uncertainty of the evidence linking these flagellate forms to the life-cycles of ruminant trypanosomes of the subgenus Megatrypanum (type

species = Trypanosoma theileri Laveran 1902), and (3) the scarcity of observations of tabanid flagellates recorded from North America.

The purposes of this study were (1) to survey for the presence of trypanosomatid parasites in tabanid species of central New York State; (2) to identify any parasites found by making morphological and biochemical comparisons with known species; (3) to differentiate monogenetic insect parasites from the developmental stages of vertebrate Trypanosoma species; and (4) to learn the mode(s) of transmission of the parasites as evidenced by the sites at which development takes place in the insect host or vector.

MATERIALS AND METHODS

I. Collection of Tabanids

Horse flies and deer flies were collected with a modified Manitoba canopy trap (Figure 3). The original trap design was described in detail and pictured by Adkins et al. (1972) and was modified for this study in the following ways:

1) Trap head (Figure 4) - Three rectangular openings were cut in the cylindrical wall of the trap head to allow cross ventilation for maintenance of living flies. The openings were covered with plastic mesh (64 openings/cm^2) to prevent the escape of the flies.

2) Target attachment - The black beach ball, used as a target, was suspended by a string fastened with a spring clasp to one of the threaded rods crossing the base of the trap head.

3) Dry ice bait - Dry ice used as bait was secured in an insect net bag and suspended by a string from the base of the trap head.

4) Black apron on canopy - A trap having a black plastic apron attached to and covering the lower two thirds of the canopy was used at some of the collecting sites (noted in I A). This black-aproned canopy was pictured by Pechuman (1972, Figure 9).

Most of the male flies and a few female flies were obtained with an insect net.

Figure 3. Modified Manitoba Canopy Trap - Collecting Site III - Ithaca, N.Y. (Tompkins Co.) Snyder Hill.

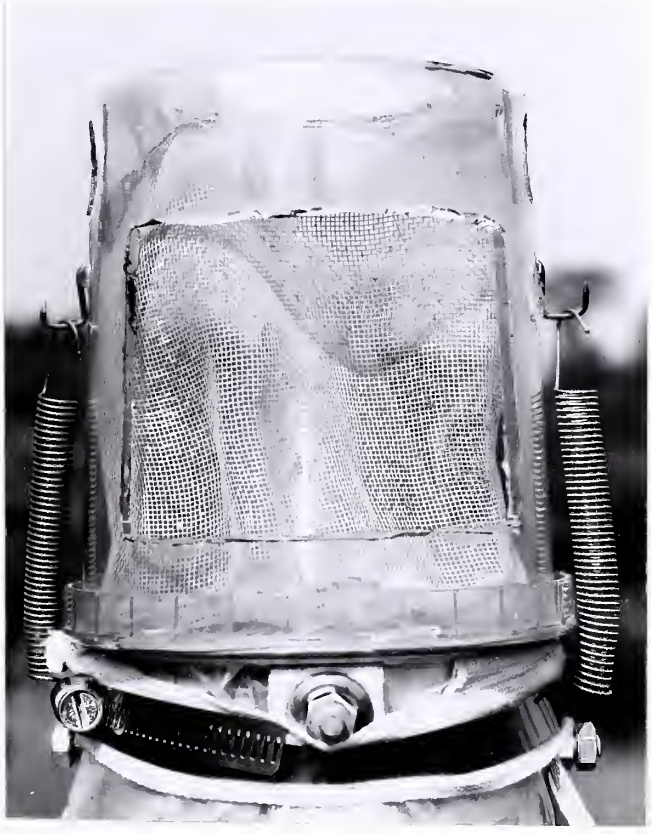


Figure 4. Modified Manitoba Canopy Trap Head.

A canopy trap was set up at least once a week from 1 June to 31 August 1972 and twice a week during the same months in 1973.

All flies were collected alive and transferred to Ball® jars (8, 16 and 32 oz.) in which the metal lid inserts had been replaced by plastic mesh (64 openings/cm^2). A piece of moist paper towel or cotton was placed in each jar and the jars were stored in an air-conditioned room (60-68° F.) or in a standard household refrigerator until the flies were dissected.

A. Descriptions of Collecting Sites

Most of the tabanids were collected at five sites (I, II, IV, V, VI). Small numbers of flies were collected at various other sites (III and "Miscellaneous Sites"). A canopy trap with a black apron was used for all collecting at Sites IV (A and B)* and "Miscellaneous Sites." Collecting at all other sites was done using a clear canopy trap or insect net.

All of the collecting sites are listed below. Sites at which deer or deer tracks were observed are marked (D). Domestic cattle were pastured within one mile of each site marked (C).

Site I - (Figure 5) - Ithaca, New York (Tompkins County) - Pond with artificial drainage, adjacent to marshy area and surrounded by young deciduous vegetation.

Site II - (D), (Pechuman, 1972, Figure 16) - Texas Hollow,

*Dr. L.L. Pechuman collected the flies from Site IV (A and B).

Figure 5. Collecting Site I - Ithaca, N.Y. (Tompkins Co.).

Town of Hector, N.Y. (Schuyler County) - Pond with artificial drainage, adjacent to marshy area and wooded area combining young deciduous and mature beech-maple-hemlock forest. This site was described in detail by Pechuman and Burton (1969).

Site III - (C), (D), (Figure 3) - Ithaca, N.Y. (Tompkins County) - Roadside adjacent to cow barns of the New York State College of Veterinary Medicine, Snyder Hill.

Site IV A - (D) - Ludlowville, N.Y. (Tompkins County) - Suburban yard, mowed lawn adjacent to alfalfa field.

Site IV B - (Pechuman, 1972, Figure 19) - Salmon Creek, Ludlowville, N.Y. (Tompkins County).

Site V - (C), (D), (Figure 6) - Ithaca, N.Y. (Tompkins County) - Pond adjacent to Schwardt Laboratory, Cornell University; surrounded by low shrubby vegetation and pasture.

Site VI - (C), (Figure 7) - Ithaca, N.Y. (Tompkins County) - Farm pond adjacent to marshy area, and low shrubby vegetation and pasture.

Miscellaneous Sites:

Jam Pond, Town of German, N.Y. (Chenango County) (Pechuman, 1972, Figure 13)

Mud Pond, Town of Oxford, N.Y. (Chenango County) (Pechuman, 1972, Figure 12)

North Lansing, N.Y. (Tompkins County)

Wildflower Preserve, Slaterville Springs, N.Y. (Tompkins County)

II. Identification of Tabanids

Tentative identification of the tabanids was made prior to dissection using the keys and descriptions in Pechuman (1972).

Figure 6. Collecting Site V - Canopy Trap and Emergence Cages - Ithaca, N.Y. (Tompkins Co.).

Figure 7. Collecting Site VI - Ithaca, N.Y. (Tompkins Co.).

After dissection, the hollowed-out integument of each fly was pinned and numbered. Definitive identifications were then made by L.L. Pechuman.

III. Dissection of Tabanids

All flies from Sites I, III, V, and VI were dissected. Samples representing all species collected from Sites II and IV (A and B) were blindly selected for dissection.

Dissections were made with the aid of 6X and 12X magnification using a Wild M-5 dissecting microscope. Patton and Cragg (1913) was used as a guide for the identification of internal structures. Dissecting needles were made by inserting minuten pins into the tips of applicator sticks. All dissecting instruments were kept in acetone when not in use during dissections. Prior to dissection, the flies were immersed in a beaker of 1:1000 zephirin chloride. This disinfected their surfaces and also immobilized the flies.

A. Dissection of Abdominal Alimentary Tract

One fly at a time was removed from the zephirin chloride and placed dorsum down on a sterile microscope slide on the microscope stage. A transverse incision was made across the first abdominal sternite using a pair of iris dissecting scissors. A longitudinal incision was then made along one pleural edge of the abdomen from one end of the first incision to the anal opening. The flap, which resulted from these right angle incisions,

was retracted with a watchmaker's forceps exposing the abdominal contents. The midgut was grasped with a forceps approximately 1 mm above the start of the ventricular bulge. The intestine was retracted and cut just above the point held by the forceps. The freed distal section of gut was then retracted posteriorly and dissected from the surrounding supporting tissues. This section of the alimentary tract was completely removed from the abdomen by severing the distal tube joining the rectum to the anus. The reproductive tract of female flies and fat bodies were removed with their supporting connective tissues by severing the vagina. A drop of sterile saline (0.85%) was placed on the free tissue specimens.

The ovaries were carefully dissected free of fat bodies and tracheal connections and kept wet for closer examination (see below). The alimentary tract was divided into midgut, hindgut and rectum. The midgut was separated from the hindgut just below the insertion of the Malpighian tubules and the hindgut was then separated from the heart-shaped rectum. Each of these intestinal sections was separately covered with sterile saline and covered with a round cover glass (18 mm circle, #1 thickness). Each section was carefully squashed by applying gentle pressure to the cover glass with the thumb.

The slide holding these preparations was transferred to the stage of an American Optical Phase Star® phase microscope and examined under low (200X) and high (450X) dry magnification. This microscope was used for all further phase microscopy. Each section was examined for the presence of flagellates and

the results recorded. If one of the gut sections was to be cultured (as in a case where flagellates were observed), the overlying coverslip was gently lifted with a forceps and the gut section with its fluid contents drawn into a sterile Pasteur pipette. This material was then transferred to a sterile tube of culture medium.

B. Dissection of Foregut and Salivary Glands

The fly carcass which remained after the abdominal contents had been removed was positioned on a sterile microscope slide such that its head was immersed in a drop of sterile saline (0.85%). The fly was grasped with two forceps. A standard dissecting forceps was used to stabilize the thorax of the fly. At the same time, a watchmaker's forceps was used to apply gentle traction to the neck of the fly. Gentle traction on the neck followed by a sharp pull resulted in decapitation and exposure of the foregut and salivary glands. The foregut was gently pulled out of the head capsule. The salivary glands were isolated by cutting the salivary ducts as close to the head capsule as possible. The salivary glands and foregut were each immersed separately in drops of sterile saline (0.85%) and covered with circular cover glasses (18 mm circle, #1 thickness) for examination. When a dilated crop was observed, it was separated by cutting its pharyngeal duct and also placed in saline under a separate cover glass for examination. Specimens to be cultured were transferred as described in Section III A.

C. Dissection of Ovaries and Determination of Parity

The ovaries in sterile saline (Section III A) were placed on a microscope slide. The ovarioles were gently teased apart using fine dissecting needles. At least two ovarioles of each ovary were isolated for examination under low (200X) dry magnification of the phase microscope. Parity determinations were made using the criteria of Thomas (1972). Ovarioles were classified as gravid if well-developed ova distending the pedicels were present. Parous flies had either ovarioles with distended pedicels indicating recent egg deposition or dilatations remaining from an older cycle of egg development. Retained ova were also noted and recorded with the above information for each fly.

IV. Examination of Blood Meals

A. Benzidine Test for Blood

A standard benzidine test for occult blood, as described by Simons and Gentzkow (1944), was used to detect blood in the alimentary tract of each fly. The gut segment or contents to be tested were absorbed on a small piece of filter paper. Tissue from each gut segment dissected was tested. Three drops of a saturated solution of benzidine dihydrochloride (in glacial acetic acid) followed by one drop of 3% hydrogen peroxide were dropped on the specimen. A dark blue-green color reaction within 60 seconds indicated that blood was present in the specimen. Human, cow, rabbit and horse blood samples (citrated) were used

periodically as controls to test the activity of the reagents.

B. Hemoglobin Crystallization Test for Blood Identity

The hemoglobin crystallization test described by Washino and Else (1972) was tried on specimens having a positive benzidine test. Human, cow, rabbit and horse citrated blood was tested in an attempt to make a reference collection of crystals from different animals.

The test was unsatisfactory for the specific identification of blood meals. Distinctive hemoglobin crystals were observed with horse blood but not with human, cow and rabbit blood samples. Tabanid blood meals, when tested, failed to exhibit any hemoglobin crystals.

V. Study of Freshly-Emerged Tabanids

A. Collection and Raising of Tabanid Larvae

Tabanid larvae were collected from the sediment at the edge of ponds (Sites I, V, VI) using a metal sieve as described by Teskey (1962). The pond sediment or earth bordering a pond was removed with a shovel and placed in the sieve. The sieve was shaken in the pond allowing the water to wash over and through the material. The sediment was sifted and turned by hand. Tabanid larvae found in the sieve were each placed in a separate (1 oz. or 2 oz.) screw-capped jar with a small amount of pond sediment. The jars were tightly capped and kept at

room temperature in the laboratory. They were examined every 24 hours for pupae or adults. Adult flies emerging in the jars were removed and dissected.

B. Collection of Freshly-Emerging Tabanids

Emergence cages made of aluminum frames (72 cm cube) covered with fine cloth mesh were used to collect adult tabanids as they emerged from pupae. Two cages were placed at the edge of a pond (Site V) with one side of each partially submerged in the water (Figure 6). Two cages were also placed in a marshy area about 10 meters from the pond (Site V, Figure 8).

The traps were examined twice a week. Any adult tabanids found in the traps were individually captured in screw-capped glass vials (2 oz.) and held for dissection (Section III A).

VI. Study of Trypanosome[1] Cultures

A. Preparation of Culture Media

Three kinds of culture media were used in this study: (1) modified N.N.N.[2] (Neopeptone) media, (2) McCoy's 5a medium (modified) and (3) CuF_{10} medium.

N.N.N. (Neopeptone) Medium. This diphasic medium, described by Wallace and Clark (1959), was used for isolation and

[1]"Trypanosome" throughout this paper refers to members of the family Trypanosomatidae; no implication concerning generic identity is intended.

[2]Nicolle, Novy and McNeal medium (Burrows, 1965).

Figure 8. Emergence Cages - Near Pond at Site V
Ithaca, N.Y. (Tompkins Co.).

maintenance of trypanosomatid flagellates. The formula for this medium appears below:

Neopeptone (Difco)	18 g.
NaCl (Allied Chemical)	4.95 g.
H_2O (distilled)	900 mls.
Agar (Difco)	18 g.
Rabbit blood	110 mls.

All further references to "N.N.N. medium" in this study refer to this medium.

Rabbit blood was obtained by cardiac puncture of New Zealand white rabbits.* The rabbits were anesthetized with Somnopentyl® (sodium pentobarbital, Pitman-Moore) (1.0 ml./5 lb. body weight i.v.) prior to cardiac puncture. Freshly drawn blood was aseptically transferred to sterile Vacutainer® tubes containing 3.8% sodium citrate and refrigerated until ready for use. A blood smear from each rabbit used was stained with Wright's stain (Section VII) and examined for parasites.

The ingredients of the N.N.N. medium were added to the water and heated until dissolved. Pyrex screw-capped culture tubes (13 X 100 mm.) were each filled with 4 ml. of the warm media. The tubes were autoclaved for 20 minutes at 17 lb./in^2. Before the media hardened, 0.5 ml. of sterile citrated rabbit blood was aseptically added to each tube. The tubes were slanted and allowed to cool. A broth was prepared using all of the above ingredients except the agar and rabbit blood. The broth

*The rabbits were caged and maintained at the Equine Isolation Building, New York State College of Veterinary Medicine, Cornell University, through the kindness of Dr. Mathias J. Kemen, Jr.

was autoclaved and when cool, 10% (v/v) of sterile citrated rabbit blood was added. This blood broth was aseptically added to each culture tube as a 0.5 ml. overlay on the hardened blood agar medium. Potassium penicillin G. (Pfizer), 200-400 units, and 15 mg. streptomycin sulfate (Pfizer) were aseptically added to each tube. The pH of the broth overlay was 7.2 (measured on a Fisher Model 520 Digital pH/ion meter). The tubes containing the N.N.N. media were refrigerated until ready for use.

McCoy's 5a Medium (Modified). This medium which was used for isolation and maintenance of flagellate cultures was supplied by Microbiological Associates Inc. and Grand Island Biological Company. The McCoy's medium, originally described by Neuman and McCoy (1958) and McCoy et al. (1959), was modified as described by Iwakata and Grace (1964).

McCoy's 5a medium (modified) was obtained as a sterile, standardized solution without serum or peptone. Each liter of this medium was supplemented with folic acid (Merck), 10 mg.; vitamin B_{12} (Sytobex®, Parke-Davis), 2.0 mg.; and $NaHCO_3$ (Malinckrodt), 2.2 g. The supplemented medium was filter sterilized by membrane filtration through a 450 mμ Seitz filter and refrigerated until ready for use.

Immediately prior to use, 4 ml. of the medium was aseptically dispensed into each 15 X 150 mm. screw-capped culture tube. The following sterilized solutions were aseptically added to each tube: 2.5% Proteose-Peptone 3 (Difco), 1.0 ml., and 0.05% hemin (Fisher), 0.1 ml. The modified McCoy's 5a medium supplemented with Proteose-Peptone and hemin was described

by Sollod and Soulsby (1968). This McCoy's 5a medium (modified) with supplementation will be referred to only as "McCoy's medium" in the remainder of this paper. Freshly prepared medium had a pH of 7.2-7.4.

CuF_{10} Medium. This medium was prepared and supplied by the New York State College of Veterinary Medicine through the courtesy of Donald H. Schlafer.

The ingredients of the medium are listed below:

fetal calf serum	50 mls.
MEM vitamin solution (Microbiological Associates)	50 mls.
penicillin (100,000 units/ml.)	2.5 mls.
streptomycin (2.0 mg./ml.)	2.5 mls.
lacto-albumin hydrolysate (LAH, 50 g./l.)	5.0 mls.
$NaHCO_3$ (44 g./l.)	5.0 mls.
H_2O (glass distilled)	385 mls.

The LAH was prepared by dissolving lactalbumin (50 g.) in 1 l. PBS (phosphate buffered saline, 0.15 M., pH 7.4) by heating to 80° C. The solution was autoclaved and then frozen. When ready for use, it was thawed and the supernatant used; the precipitate was discarded. Unused portions were refrozen.

B. Inoculation and Maintenance of Cultures

Tissues to be cultured were aseptically transferred to fresh culture tubes. The tubes were slanted in racks and incubated at 28° C. in a Will Scientific water-jacketed incubator or at 37° C. in an Electric Heat Control Apparatus Co. incubator, depending on the design of the experiment. Uninoculated tubes were incubated as controls. All of the other tempera-

tures mentioned in this paper are given in degrees Celsius (°C) unless otherwise noted.

Cultures were examined microscopically at least once a week. Living cultures were examined using a phase microscope. Morphological determinations were made from living as well as stained specimens (Section VIII). Viable cultures were transferred to fresh media at least once every two weeks.

C. Culture Counts

All counts were made using an American Optical Bright-Line Hemocytometer. The culture to be counted was thoroughly mixed using a Pasteur pipette. A drop was then removed and placed in a Syracuse watch glass.

A white cell pipette was used to dilute the culture fluid 20 X with sterile saline (0.85%). Both sides of the hemocytometer were filled with the fluid to be counted. Five 1 mm. squares were counted on each side of the hemocytometer. The volume of fluid counted over each 1 mm. square was 0.1 mm^3. Therefore, the number of cells per mm^3 was calculated using the following formula:

$$\frac{\text{flagellates X 20 (=dilution) X 10}}{\text{10 (= \# 1 mm. squares counted)}} = \frac{\text{\# flagellates}}{mm^3}.$$

VII. Trypanosome Stains

Two kinds of stain were used to aid morphological examination of blood, fly tissues and cultures for flagellates:

Wrights' stain and Giemsa stain. Specimen slides to be examined were air dried, fixed in Ponselle's fixative (methanol, 5 parts; tincture of iodine, 1 part) for 5 minutes and rinsed with methanol (Wallace, 1962).

Giemsa Stain. Slides were stained using the Triton-Giemsa technique described by Burrows (1965). After staining the slides in Giemsa stain (Allied Chemical) with added Triton X-100, they were rinsed in buffered water, pH 7.0-7.2, for 5 minutes and then allowed to dry. Euparol was used as a mounting medium.

Wright's Stain. Slides were stained with Wright's stain (Allied Chemical) using the technique described by Burrows (1965). Euparol was used as a mounting medium.

VIII. Trypanosome Measurements

Living and stained flagellates were measured with the aid of an ocular micrometer disc (B+L) inserted in the eyepiece of a phase microscope. The micrometer was standardized using a stage micrometer (B+L).

Estimation of Trypanosome Sizes. Estimates of the size of living parasites in each culture were made weekly or biweekly. The parasites were viewed using phase microscopy. The approximate lengths (L) of the flagellates in five microscopic fields (at 450X magnification) of each culture were recorded.

Actual Measurement of Trypanosomes. Giemsa-stained preparations of fixed flagellates were measured using light micro-

scopy. Measurements were made at 1000X (oil immersion) magnification.

The following measurements were made: free flagellum length (F), distance from posterior end of cell to kinetoplast (PK), distance from posterior end of cell to middle of nucleus (PN), distance from middle of nucleus to anterior end of cell (NA), and total length (L) (see Figure 9). These values were used to calculate the nuclear index (NI = PN/NA) and the kinetoplastic index (KI = PN/KN) as described by Hoare (1972).

If NI = 1, the nucleus is in the middle of the cell; if NI < 1, the nucleus is in the posterior portion and if NI > 1, the nucleus is in the anterior portion of the cell (Hoare, 1972). Similarly, Keymer (1967) pointed out that if KI = 2, the kinetoplast is halfway between the nucleus and the posterior end of the cell; if KI < 2, the kinetoplast is nearer to the posterior end than to the nucleus and if KI > 2, the kinetoplast is nearer to the nucleus than to the posterior end of the cell.

IX. Frozen Storage of Trypanosomes

An aliquot (0.5-1.0 ml.) of each culture to be frozen was aseptically transferred to a sterile screw-capped vial (2 dram). Sterile glycerol (Mallinckrodt) was added equivalent to 10% of the volume. The vials were frozen at -70° in a Revco.

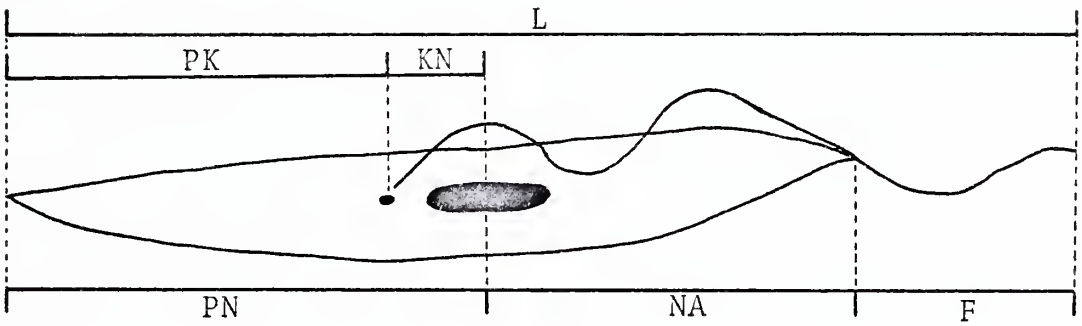


Figure 9. Measurements of Trypanosomatid Flagellate (after Hoare, 1972).

PK = distance between posterior end and kinetoplast
PN = from posterior end to middle of nucleus
KN = from kinetoplast to middle of nucleus
NA = from middle of nucleus to anterior end
F = length of free flagellum
L = total length of flagellate (PN+NA+F)

X. Preparation of Clones

Two methods of preparing clone cultures were tried. Only sterile materials were used and all transfers were aseptically performed. The first method was a modification of the dilution technique used by Inoki (1960). A drop of an actively growing culture to be cloned was transferred to a depression slide using a Pasteur pipette. Additional depression slides were each filled with individual drops of culture media (N.N.N. or McCoy's). The original drop was transferred to one of the culture fluid drops and then in turn a drop was taken from this slide well and diluted in a second drop of medium. This dilution process was repeated a few times and, after each dilution, a drop was examined using phase microscopy (200X). A drop which was finally found to harbor only a single flagellate was transferred to a fresh culture tube and incubated.

The second method was a modification of the divided dilution method of Weinman (1972). A drop of a culture was diluted as above and examined periodically either in a depression slide or between two cover slips. The drop observed to have only a single flagellate was transferred to a tube containing 2.5 mls. of the appropriate culture medium. Half a ml. of this fluid was then transferred to each of 5 culture tubes which were incubated. If flagellates grew in only one of the five tubes, the viable culture was regarded as a clone.

XI. Type Cultures

Trypanosoma (Megatrypanum) theileri Laveran 1902. A type culture of Trypanosoma theileri Laveran 1902 was purchased from the American Type Culture Collection, Bethesda, Maryland. The culture obtained (ATCC #30,017) was originally isolated from a mature dairy cow at the University of Pennsylvania in 1966 (Sollod and Soulsby, 1968). Two frozen stabilates of the culture (July, 1969 and February, 1970), which had been passed 160 times, were obtained. The July, 1969 stabilate was thawed rapidly and inoculated into McCoy's medium at 37°. The culture was maintained under these conditions with transfers every 4 to 5 days.

T. theileri-like Trypanosomes. A culture of trypanosomes (CuF_{10} at 37°) isolated from a dairy cow at the New York State College of Veterinary Medicine was examined.*

Trypanosoma (Herpetosoma) lewisi (Kent) Laveran and Mesnil. A white rat infected with Trypanosoma lewisi was obtained from the Carolina Biological Supply Company. Phase microscopic examination of a sample of blood from the rat's tail revealed living trypanosomes morphologically consistent with T. lewisi. Four to five milliliters of blood were removed from the rat by cardiac puncture after anesthetizing the animal with diethyl ether. The blood was cultured in N.N.N. medium at 28°. The cultures were transferred to fresh media once a week. After 3 weeks, the cultures were transferred to McCoy's medium at 28°

*This culture was provided through the courtesy of Donald H. Schlafer.

and maintained under these conditions with transfers at least once every two weeks.

XII. Artificial Feeding of Tabanids

The chorioallantoic membrane feeding procedure described by Wallis (1969) was attempted with tabanids. To test the method, one female Hybomitra lasiophthalma was placed in a 16 oz. Ball jar in which the metal insert had been replaced with plastic mesh (64 openings/cm^2). An evacuated chicken egg, containing 10% sucrose colored with blue vegetable dye, was placed on top of the jar so that the intact chorioallantoic membrane was resting on the plastic mesh. The jar was left at room temperature for 18 hours (8 hours of darkness), the fly removed and the alimentary tract dissected (Section III A). The midgut of the fly contained a blue fluid which resembled the dye solution given.

The same method was used in an attempt to feed actively growing flagellate cultures to female tabanids which had just emerged from pupae in the laboratory. Five freshly-emerged Chrysops species were placed in individual Ball jars. Aliquots of T. theileri type culture (Section XI) grown in McCoy's medium at 37° were placed in each egg shell used for feeding. The flies expired within 2-6 days after being placed in the jars. Each dead fly was dissected immediately after it was found dead. There were no indications that the flies had fed on the culture fluid. All of the flies were nulliparous and none of them were

infected with flagellates. A freshly-emerged C. vittatus was given the opportunity to feed on a culture of choanomastigotes (Crithidia sp.). On dissection, the fly was found to be nulliparous and no flagellates were observed.

Attempts to feed culture fluid containing flagellates to flies were unsuccessful; however, one tabanid imbibed a sucrose and dye solution, indicating that this method may be used to feed tabanids in the laboratory.

XIII. Sampling of Deer for Trypanosomes*

Five white-tailed deer from the herd maintained at the New York State Game Farm, Ithaca, New York, were irregularly sampled for the presence of trypanosomes. The deer to be sampled were immobilized with a tranquilizer (succinyl choline) dart pistol. The deer were bled from the jugular vein using Vacutainer® needles and heparinized tubes.

The fresh heparinized blood samples were examined for flagellates as thin wet smears and fixed Wright-stained smears. The blood was cultured in N.N.N. medium and McCoy's medium at both 28° and 37°. The cultures were examined every few days for growth of flagellates and viable cultures, transferred every 7-10 days.

*The procedures for procuring blood samples which are outlined in this section were carried out by Dr. Frederick L. Jacobsen and members of the staff of the New York State Game Farm, Ithaca, New York.

XIV. Serological Studies of Trypanosomes

Various immunological techniques were tried in an attempt to compare tabanid flagellates with known species. By comparing the serological reactions of tabanid flagellates with those of T. theileri and T. lewisi, it was hoped that further information could be obtained about the taxonomic identity of the insect flagellate species. The procedures and the results of these trials are described below.

A. Preparation of Antiserum in Rabbits Using Tabanid Trypanosomes as Antigens

New Zealand white rabbits were inoculated intravenously with antigens (Ag) prepared from clone cultures of trypanosomatid flagellates from Tabanus quinquevittatus. The clone cultures used as the source of Ag were counted (Section VI C). The rabbits were bled for serum at regular intervals following the inoculations. Blood was obtained by cardiac puncture or vacuum flask suction from the marginal ear vein. Control bleedings were made prior to each set of Ag inoculations. The details of Ag preparation will be discussed.

Preparation of Antigen. Antigens consisted of actively growing clone cultures of trypanosomes. The first set (Ag I) of three cultures for Ag use were grown in N.N.N. medium. Each original Ag I culture contained approximately 50,000 flagellates. To separate the trypanosomes from red blood cells, the cultures were centrifuged (International Equipment Co. centri-

fuge) at 550-600 RPM for 20 min. The cultures were decanted and the supernatant spun at 1850 RPM for 15 min. After decanting, the sediment was washed twice with 0.9% physiological saline (spun down at 1850 RPM for 15 min. each time) and resuspended in 0.85 ml. aliquots of 0.9% physiological saline. Examination of this Ag I using phase microscopy revealed some scattered pieces of cells and debris.

The second and third sets of cultures for Ag use were grown in McCoy's medium. Active cultures were pooled to achieve the greatest concentration of flagellates. Media from these cultures were used as Ag II and Ag III. No special preparation of the fluid was made.

Schedule of Antigen Inoculations and Antiserum Collections. The schedule of i.v. injections and bleedings is given in Table II. Citrated blood samples taken from each rabbit on Day 18 of the Ag II series were examined for the presence of living flagellates. No living or dead organisms were observed.

B. Antiserum from T. theileri-like Antigens

Serum from Rabbit.* A New Zealand white rabbit was inoculated intramuscularly with CuF_{10} culture fluid containing approximately 400,000 T. theileri-like trypanosomes cultured from a cow. Ten days following this inoculation, serum was collected for study.

*These procedures including the collection of serum for antiserum study were done by Donald H. Schlafer, New York State College of Veterinary Medicine.

Table II. Schedule of Ag Inoculations and Antiserum Collections.

Day	Rabbit	Ag Given	Ag[1]	Serum Obtained
0	BC,DE	AgIa, 0.65 ml	disrupted	Control
7	"	AgIb, 0.75 ml	"	-
14	"	AgIc, 0.75 ml	"	-
17	DE[2]	-		Bleeding (BL) I
21	DE	-		BL II
28	DE	-		BL III
0 (48[3])	DE,HI	AgIIa, 1.0 ml	1000	Control
1	"	AgIIb, 1.0 ml	1000	-
7	"	AgIIc, 0.6 ml	6000	-
14	"	AgIId, 1.0 ml	1000	-
18	"	-		BL I
21	"	-		BL II
28	"	-		BL III
34	"	-		BL IV
0 (58[3])	DE,HI	AgIIIa, 1.0 ml	3600	Control
5	"	AgIIIb, 0.8 ml	9400	-
20	"	AgIIIc, 1.8 ml	2800	-
28	"	AgIIId, 2.0 ml	1500	-
35	"	-		BL I

[1]# flagellates/mm[3]

[2]On Day 16, Rabbit BC died of pneumonia.

[3](#) = Day from start of AgI series.

Serum from Cow[1]. Blood was collected for antiserum study from the jugular vein of a heifer which had a positive culture (CuF_{10}) for T. theileri-like trypanosomes.

Serum from Deer. Blood was collected for antiserum study from three deer at the New York State Game Farm[2]. These serum collections were made in conjunction with the collections of heparinized blood for flagellate culture studies (Section XIII).

C. Agglutination Test

Test with Live Trypanosomes. A modification of the slide agglutination test described by Cunningham and Vickerman (1962) was used. The antiserum used was prepared from rabbits injected with tabanid flagellates (Section XIV A) and a rabbit injected with cow trypanosomes (Section XIV B). In addition, plasma from two cows infected with trypanosomes were tested. Two-fold serial dilutions of antiserum (or cow plasma, in two cases) from ½ to 1/4096 were tested against samples of living trypanosome cultures. These cultures (Sections VI, X, XI) which served as antigens, included a type culture of T. theileri, a clone culture of flagellates from Tabanus quinquevittatus and a culture of T. lewisi. All cultures tested were grown in McCoy's medium. Samples of cultures to be tested were counted with a hemocytometer (Section VI C). The concentration of flagellates in each culture were standardized by dilution

[1]Collected by Donald H. Schlafer.

[2]Collected by Dr. Frederick L. Jacobsen.

with McCoy's medium.

Normal sera collected from the rabbits before they were inoculated for antiserum production served as control sera. Serial dilutions of the control sera from ½ to 1/4096 were tested. All serum dilutions were made using PBS. Additional controls tested against the trypanosome cultures included: neutral buffers of different molarities, bovine serum albumin and buffers from pH 4.0 to 10.0.

One drop of each serum dilution (or control solution) to be tested was placed in a ring of a microflocculation slide (Clay Adams). Each slide had 12 rings, each of 12 mm. internal diameter. One drop of the culture (Ag) to be tested was added to each serum or control dilution. The slides were gently agitated and incubated at 28° for 24 hours in a moist chamber to prevent evaporation. After 24 hours, the individual rings were examined under light microscopy (200X) and the agglutination reactions graded.

Tabanid clone cultures, T. theileri type cultures and T. lewisi cultures, when tested against rabbit sera from animals given Ag inoculations, failed to demonstrate the presence of anti-trypanosome antibodies. Sera taken from rabbits at different intervals after Ag inoculations failed to produce different agglutination patterns from those obtained with control (pre-Ag inoculation) sera. Table III summarizes the results of testing living trypanosome cultures vs. rabbit sera. Each line represents the average results from the tests against sera in each of the three series of Ag inoculations. No significant

Table III. Agglutination Tests with Cultured Flagellates

Ag		Antisera Dilutions											
		2	4	8	16	32	64	128	256	512	1024	2048	4096
Tabanid flagellates (*T. quinquevittatus* clone)													
Control (+)*													
Serum from Bleeding	0	-	-	+	+	+	+	+	+	+	+	+	+
" " "	I	-	-	+	+	+	+	+	+	+	+	+	+
" " "	II	-	-	-	-	+	+	+	+	+	+	+	+
" " "	III	-	-	-	-	+	+	+	+	+	+	+	+
" " "	IV	-	-	-	+	+	+	+	+	+	+	+	+
T. theileri (type culture)													
Control (-)													
Serum from Bleeding	0	-	-	-	±	±	±	-	-	-	-	-	-
" " "	I	-	-	-	±	±	±	-	-	-	-	-	-
" " "	II	-	-	-	±	±	±	-	-	-	-	-	-
" " "	III	-	-	-	±	±	±	-	-	-	-	-	-
" " "	IV	-	-	-	±	±	±	-	-	-	-	-	-
T. lewisi													
Control (+)													
Serum from Bleeding	0	-	-	+	+	+	+	+	+	+	+	+	+
" " "	I	-	+	+	+	+	+	+	+	+	+	+	+
" " "	II	-	+	+	+	+	+	+	+	+	+	+	+
" " "	III	-	-	+	+	+	+	+	+	+	+	+	+
" " "	IV	-	-	+	+	+	+	+	+	+	+	+	+

*Control = Ag vs. PBS; (+) indicates agglutination; (-) indicates no agglutination; (±) means 50% of the tests exhibited agglutination.

differences in agglutination were seen. Table III indicates that the agglutination test was unsatisfactory for comparing different species of trypanosomes.

All cultures of clone flagellates and T. lewisi flagellates exhibited autoagglutination to the same degree as agglutination in PBS alone. However, a comparison of agglutination occurring in serum vs. agglutination in PBS indicates certain differences. Samples of tabanid clones and T. lewisi cultures at low dilutions (1:2 to 1:16) exhibited less agglutination in serum than in PBS (Table III). This may have been caused by serum protein inhibition of flagellate protein-protein interactions. Trypanosomes from T. theileri type cultures exhibited no autoagglutination or agglutination in PBS. However, greater agglutination was noted in serum than in PBS alone in half of the tests. Agglutination occurred in the middle range of dilutions (1:16 to 1:64) (Table III). This non-specific agglutination of cultured forms of T. theileri has been reported with sera from cattle, horse, sheep, dog, and goat at dilutions of 1:10 to 1:80 (Trautmann, 1922 as reported by Mesnil, 1923).

To eliminate autoagglutination of cultured flagellates, culture samples were diluted and exposed to buffers of different pHs and salt concentrations. Flagellate samples at dilutions of 1:1 to 1:10 in PBS (0.15 M, pH 7.4) all exhibited the same degree of agglutination as undiluted controls. Culture samples placed in buffer solutions which varied by one pH unit from pH 4.0 to 10.0 all exhibited the same degree of agglutination as controls at pH 7.4. Culture samples in buffered salt

solutions (pH 7.4) of molarities 0.15 M, 0.25 M and 0.5 M all exhibited the same degree of agglutination as the original undiluted samples. Bovine serum albumin (5 mg/ml) in dilutions of 1:1 with culture samples produced no change in agglutination from undiluted culture samples.

Since changes in the test solutions had no effect on autoagglutination, a procedure to modify the surface proteins of the flagellates was attempted. Trypanosomes from tabanid clone, T. lewisi and T. theileri cultures were trypsinized and fixed with formalin as described below.

Test with Trypsinized Trypanosomes. Slide agglutination tests (Section XIV C) were carried out with trypanosomes treated with trypsin using a modification of the method of Vattuone and Yanovsky (1971).

Cultures to be tested (T. theileri, cloned tabanid flagellates, T. lewisi) were centrifuged at 10,000 g for 15 min. at 5° in a Sorvall RC2B, decanted, and resuspended in 5 mls. PBS. The tubes were recentrifuged at 10,000 g for 15 min., decanted and each resuspended in 2 mls. PBS (to obtain a concentration of approximately 3 g% of wet weight). A sample (0.2 ml) from each tube was used as an untreated control. Formaldehyde (2%, 0.2 ml.) was added to each of the control samples. The tubes with the 3 g% flagellates were centrifuged at 10,000 g for 15 min. and decanted. Trypsin, 1-250 (hog pancreas, Nutritional Biochemicals), 2 mls. of a 4 mg./ml. solution, was added to each pellet (approximately 20:1 v/v) and the tubes incubated

at 37° for 45 min. Trypsin inhibitor (egg white, B grade, Calbiochem), 2 mg./mg. trypsin, was added to each tube to terminate the reaction. The treated flagellates were centrifuged at 10,000 g for 15 min., suspended in 4 mls. PBS, centrifuged again and resuspended in 1.8 mls. PBS. Formaldehyde (1.8 mls. 2%) was added to each tube. The concentration of flagellates in each tube was determined by counting with a hemocytometer (Section VI C) and then standardized by dilution with PBS. These standardized, trypsinized, fixed flagellates were used as Ag in slide agglutination tests (Section XIV C).

Trypsinized flagellates showed the same degree of autoagglutination as untreated flagellates. However, when tested against rabbit sera, treated flagellates showed agglutination patterns different from those of untreated controls. Trypsinized flagellate samples tested with rabbit sera and cow plasma from two infected animals showed the same agglutination patterns as control trypsinized samples. The decrease in agglutination at low serum dilutions noted with untrypsinized clone and T. lewisi flagellates (Table III) was not observed with trypsinized forms. Similarly, the agglutination at middle serum dilutions noted with untrypsinized T. theileri trypanosomes was not observed with trypsinized forms.

Despite the slight differences in agglutination patterns obtained with trypsinized and untrypsinized flagellates, the agglutination test was unsatisfactory for comparing flagellates.

D. Immuno-Gel-Diffusion Test

Agar-gel-diffusion tests were run using a modification of the method of Splitter et al. (1967). Antisera tested were the same as those used in the agglutination tests (Section XIV C). In addition, serum collected from a cow infected with trypanosomes and sera collected from white-tailed deer were used (Section XIV B). All antisera were frozen at -70° in a Revco until ready for use.

Preparation of Antigen. Antigens were prepared from flagellate cultures grown in McCoy's medium (T. theileri at 37°, cloned tabanid flagellates and T. lewisi at 28°) (Sections VI, X, XI). Cultures were centrifuged at 2000 RPM (International Equipment Co. centrifuge) for 20 min. and washed twice with PBS at room temperature. Each pellet (flagellates) was resuspended in 2 mls. PBS and dialyzed at 4° versus 0.05 M. Tris buffer, pH 8 (3 changes: 2 l. for 1 hr., 1 l. for ½ hr., 2 l. for 1½ hr.). The dialyzed cultures were frozen and thawed and then disrupted at 4° with sonic oscillation (Sonifier Cell Disruptor Model W140) using a micro tip (maximum setting) for 2-5 min. The sonicated samples were frozen and thawed twice, stirred for 15 hrs. at 4° and frozen in 0.5 ml. aliquots until ready for use as antigens. Selected samples were concentrated using a Virtis lyophilizer.

Immunodiffusion Procedure. Slides were prepared for demonstration of double diffusion precipitin reactions. Ionagar No. 2 prepared with 0.02% sodium azide (Fisher) in Tris buffer, pH 7.6 (Trizma, Sigma) was used to cover the microscope slides.

Each slide was covered with 1 ml. 1% agar and allowed to dry. An overlay of 2 mls. 2% agar was then added to each slide. After drying, wells, 4 mm. in diameter, were cut in the agar-gel. Six wells, in an hexagonal arrangement, were each positioned 3 mm. from the center well. Slides with two sizes of center wells (4 mm. and 6 mm.) were used in the precipitin tests.

The antigen to be tested was placed in the center well of each hexagon using a Pasteur pipette and the antisera were placed in the surrounding wells. The slides were incubated at 28° in a moist chamber for 24 hours. The wells were refilled twice during incubation. Results were read after 24 hours.

Agar-gel immunodiffusion tests failed to demonstrate the presence of anti-trypanosome antibodies. Flagellate antigens prepared from tabanid clone cultures (T. quinquevittatus), T. lewisi and T. theileri cultures when reacted with sera (and plasma) from naturally infected cattle and deer and sera from inoculated (i.v. and i.m.) rabbits failed to exhibit precipitin lines on the test slides. Therefore, no comparisons of T. theileri, T. lewisi, and tabanid flagellates could be made using this test method.

Rabbit serum from one rabbit inoculated (i.m.) with cow trypanosomes grown in CuF_{10} medium (containing fetal bovine serum), when reacted with deer and cow sera, produced precipitin lines. The rabbit had apparently produced antibodies to the bovine serum proteins found in the culture fluid (CuF_{10}). These antibodies, when reacted with cow and deer test sera, produced precipitating Ag-Ab complexes. This result indicated

that, though the test system was suitable for demonstrating the reactions of precipitating antibodies, no such anti-trypanosome antibodies could be observed.

E. Electrophoresis of Trypanosome Antigens

Protein fingerprinting of the components of antigens prepared from tabanid flagellates, T. theileri and T. lewisi was attempted using cellulose acetate electrophoresis (Gelman Instrument Co., 1970). Although control samples of the antigens were successfully stained with Coummassie blue dye, no separation of protein components was achieved.

F. Passive Hemagglutination Test

Sheep blood in Alsever's solution (1:1)* was the source of red blood cells (rbc). The Ag (disrupted, concentrated flagellates) and antisera used were the same as those used in the immunodiffusion tests (Section XIV D). In addition, two cultures of choanomastigote flagellates (from Tabanus quinquevittatus and T. marginalis) were used as sources of Ag. The protein concentration of the Ag was measured using the biuret method described in Campbell et al. (1970). The standard for these measurements was Moni-trol I (albumin) (B5103, Dade, Div. Amer. Hospital Supply Corp.). Complement was destroyed by incubating

*The sheep blood was provided by Dr. J.T. Wang, New York State College of Veterinary Medicine, Cornell University.

antisera in a 56° water bath for 1 hour prior to use. Heterophile antibodies were removed by incubation of antiserum with 25 - 30% washed, packed sheep rbc at 37° for 30 min. prior to use. Normal rabbit serum at 1:100 dilution was used as a stabilizing protein.

Two different coupling agents were tested for their abilities to achieve hemagglutination reactions: tannic acid and glutaraldehyde.

Tannic Acid as a Coupling Agent. The method for tanning rbcs described by Campbell et al. (1970) was used. Tannic acid (0.005%) (Baker) was coupled to washed sheep rbcs. Individual aliquots of 0.05 ml. packed tanned cells were each sensitized with 30 mg. of antigen at room temperature for 10 min. or at 37° for 30 min.

Serial dilutions of antiserum from ½ to 1/4096 were prepared in 15 X 150 mm. glass test tubes. Tanned sensitized rbcs suspended in PBS and rabbit serum (1:100) were added to the antiserum dilutions. The suspension of sensitized rbcs was added to each antibody dilution in the proportion 1:10 (v/v). The sensitized rbcs comprised 2 - 3% of the volume of each tube. Controls included sensitized rbcs versus diluent (PBS + rabbit serum), sensitized rbcs versus fetal bovine serum (30 mg. protein/ml.) and non-sensitized tanned rbcs versus the antisera. The tubes were gently agitated and refrigerated at 4° for 24 hours. Hemagglutination reactions were scored with the naked eye and with the aid of light microscopy (100 X magnification).

Glutaraldehyde as a Coupling Agent. The method used for

coupling rbcs with glutaraldehyde and sensitizing the cells with Ag was described by Avrameas et al. (1969). Individual 1.0 ml. aliquots of packed sheep rbcs (in suspension) were each sensitized with 30 mg. of antigen in the presence of 1.0 ml. of a 2.5% aqueous solution of glutaraldehyde (Ladd) by gentle mixing at room temperature for 2 hours.

The antiserum dilutions, controls and procedures for observing hemagglutination were identical to those used with tannic acid sensitized rbcs (see above).

Hemagglutination patterns with test antisera were the same as those with control sera. Tests with tannic acid and glutaraldehyde as coupling agents failed to demonstrate the presence of anti-trypanosome antibodies in sera from infected animals. Therefore, the passive hemagglutination method was ineffective for comparing T. theileri, T. lewisi and tabanid flagellates.

RESULTS

I. Tabanid Collections

Tabanids were collected from 5 June to 31 August 1972 and 1973. Collections were made at Sites I, II, III and IV (A and B) in 1972 and at Sites II, V and VI in 1973. Small numbers of flies were collected at "Miscellaneous Sites" (Materials and Methods, IA) during both summers.

A. Tabanids Infected with Trypanosomes

The total number of female tabanids collected at all sites using a canopy trap (or insect net) and their infection rates are listed in Table IV. Since no male flies were infected, all references to tabanids refer to female specimens unless otherwise noted. Infected flies include specimens in which flagellates were seen at dissection as well as those from which flagellates were cultured. Infected flies harbored immotile and/or motile flagellates. The morphology of these forms will be described in a later section (II A).

Table V A lists the species infected with flagellates and those from which flagellates were cultured. The only species in which flagellates were not observed at dissection but from which positive cultures were obtained was Hybomitra zonalis. In addition to the species in Table V A, the species in Table V B were examined but were not infected. A comparison of

Table IV. Tabanid Infection Rates

Tompkins County

# Collected*			# Infected			% Infected		
1972	1973	'72&'73	1972	1973	'72&'73	1972	1973	'72&'73
253(+6)	255(+22)	508(+28)	36	92(+1)	128(+1)	14(0)	36(5)	25(4)

Outside Tompkins County

# Collected			# Infected			% Infected		
1972	1973	'72&'73	1972	1973	'72&'73	1972	1973	'72&'73
54(+23)	(+28)	54(+51)	19(+8)	(+16)	19(+24)	35(35)	(57)	35(47)

Total

# Collected			# Infected			% Infected		
1972	1973	'72&'73	1972	1973	'72&'73	1972	1973	'72&'73
307(+29)	255(+50)	562(+79)	55(+8)	92(+17)	147(+25)	18(28)	36(34)	26(32)
336	305	641	63	109	172	19%	36%	27%

*Numbers in parenthesis (+#) refer to flies collected with an insect net; all other flies were collected in a canopy trap.

Table V A. Tabanid Species Infected with Trypanosomes

Species	Flagellates Observed		Flagellates Cultured	
	1972	1973	1972	1973
Chrysops ater Macquart		X		X
C. callidus Osten Sacken		X		
C. cincticornis Walker	X		X	
C. cuclux Whitney		X		X
C. geminatus Wiedemann	X		X	
*C. indus Osten Sacken	X		X	
*C. macquarti Philip (N)	X		X	
C. mitis Osten Sacken		X		X
*C. shermani Hine (N)	X		X	
C. vittatus Wiedemann	X	X	X	X
*Hybomitra difficilis (Wiedemann)	X		X	
*H. epistates (Osten Sacken)	X	X		X
H. illota (Osten Sacken)	X	X	X	X
H. lasiophthalma (Macquart)	X	X	X	X
H. sodalis (Williston)	X		X	
H. typhus (Whitney)		X		X
*H. zonalis (Kirby)			X	
Tabanus calens Linnaeus	X	X		X
T. marginalis Fabricius		X		X
T. quinquevittatus Wiedemann	X	X	X	X
T. sackeni Fairchild	X			
T. similis Macquart	X	X	X	X
T. sparus milleri Whitney	X	X	X	X
T. sulcifrons Macquart	X	X	X	X
T. superjumentarius Whitney	X	X	X	X
TOTALS:				
Species	18	16	16	15
Species Examined	30	24	19	16
% Species Infected	60%	67%		

*Infected flies collected outside Tompkins County only.
(N) indicates that infected flies were collected with insect net exclusively.

Table V B. Tabanid Species Not Infected with Trypanosomes

Atylotus bicolor (Wiedemann) (3C)
A. thoracicus (Hine) (5N)

Chrysops calvus Pechuman and Teskey (3)
C. frigidus Osten Sacken (1)
C. niger Macquart (2)
C. univittatus Macquart (2)

Hybomitra aurilimba (Stone) (2)
H. microcephala (Osten Sacken) (1)

Tabanus fairchildi Stone (1C)
T. lineola Fabricius (3)
T. nigripes Wiedemann (1)

(#) indicates number of specimens examined.

(C) indicates species cultured with negative results.

(N) indicates species collected with an insect net.

Tables V A and V B shows that 25 of the 36 tabanid species collected during both summers, or 69% of the species were infected.

Most of the tabanids collected in Tompkins County comprised four species. These species are listed in order of their seasonal occurrence (cf. Pechuman and Burton, 1969) with their infection rates in Table VI. There were higher percentages of infection in the late flying species than in early ones. Immotile flagellate forms were often difficult to detect so that the overall lower infection rates in 1972 were probably due to a lack of recognition of these forms early in the study. One third of the blooded flies collected in 1972 were infected and all of the blooded flies in 1973 were infected. This discrepancy may again be due to a recognition error; also, the sample size of blooded flies which was twice as large in 1972 as 1973.

Additional species collected in Tompkins County with their infection rates are listed in alphabetical order in Tables VII (Chrysops spp.), VIII (Hybomitra spp.) and IX (Tabanus and Atylotus spp.). Table VII indicates that the same percentage of Chrysops specimens were infected during both summers. Two blooded Chrysops specimens collected were infected. The Hybomitra species (Table VIII) exhibited the lowest percentage of infection. With the exception of T. marginalis, which was infected with a flagellate form different from the other Tabanus species (Section II A), T. sparus milleri had the highest infection rate among the species in Table IX. The latter species is known to be an aggressive bloodsucker of man and animals

Table VI. Most Abundant Tabanid Species Collected[1] in Tompkins County

Species	# Collected		# Infected		% Infected	
	1972	1973	1972	1973	1972	1973
H. lasiophthalma	69(0)[2](+3)	53(1)(+14)	1(0)	8(1)	1%	15%
T. similis	53(1)(+2)	39(0)(+2)	4(0)	9(0)	8%	23%
T. quinquevittatus	58(11)	68(6)	16(4)	31(6)	28%	46%
T. sulcifrons	9(6)	39(2)	2(2)	24(2)	22%	62%
TOTALS:	189(+5)	199(+16)	23	72	12%	36%

[1]All tabanids were collected with a canopy trap except those listed after a "+" in parenthesis (+#). The latter were collected with an insect net.

[2]Underlined numbers in parentheses (#) refer to the number of specimens exhibiting a positive benzidine test for blood. Each parenthesis follows the group of flies to which the enclosed number applies.

Table VII. *Chrysops* Species Collected[1] in Tompkins County

Species	# Collected		# Infected		% Infected	
	1972	1973	1972	1973	1972	1973
C. ater		8		2		25%
C. callidus	3	8(+1)	0	3	0%	38%
C. calvus	3		0		0%	
C. cincticornis[2]	3	1	2	0	67%	0%
C. cuclux		1		1		100%
C. frigidus		(+1)		(0)		0%
C. geminatus[2]	5(1)		3(1)		60%	
C. indus[2]	2(+1)	4(+2)	0	0	0%	0%
C. macquarti[2]	5		0		0%	
C. mitis		4(+1)		1		25%
C. niger	2		0		0%	
C. univittatus	1	1	0	0	0%	0%
C. vittatus[2]	4(1)	1	2(1)	1	50%	100%
TOTALS:	28(+1)	28(+5)	7	8	25%	28%

[1]Notations concerning flies collected by different methods and number of blooded flies are the same as those used in Table VI.

[2]Specimens of these species collected outside of Tompkins County were also infected, as was *C. shermani* (Table X).

Table VIII. *Hybomitra* Species (excluding Table VI) Collected in Tompkins County[1]

Species	# Collected		# Infected		% Infected	
	1972	1973	1972	1973	1972	1973
H. aurilimba	1	1	0	0	0%	0%
H. illota[2]	5	5	1	2	20%	40%
H. microcephala		1		0		0%
H. sodalis	11(1)	3	2(0)	0	18%	0%
H. typhus	1	2	0	1	0%	50%
TOTALS:	18	12	3	3	17%	25%

[1]All flies were collected in a canopy trap. Notations concerning number of blooded flies are the same as those used in Table VI.

[2]Specimens of this species collected outside of Tompkins County were also infected, as were *H. difficilis*, *H. epistates*, *H. zonalis* (Table X).

Table IX. *Tabanus* and *Atylotus* Species (excluding Table VI) Collected in Tompkins County[1]

Species	# Collected 1972	# Collected 1973	# Infected 1972	# Infected 1973	% Infected 1972	% Infected 1973
T. calens	11(8)	4	1(0)	2	9%	50%
T. fairchildi	1		0		0%	
T. lineola	1	2	0	0	0%	0%
T. marginalis		1(+1)		1(+1)		100%
T. sackeni	1		1		100%	
T. sparus milleri[2]	1	6	0	5	0%	83%
T. superjumentarius	3	2	1	1	33%	50%
TOTALS:	18	15(+1)	3	9(+1)	17%	60%
Atylotus bicolor		1		0		0%

[1]Notations concerning the flies collected by different methods and number of blooded flies are the same as those used in Table VI.

[2]Specimens of this species collected outside Tompkins County were also infected.

during its flight season (Pechuman, 1972). Table X lists all species collected outside of Tompkins County with their infection rates. C. indus, C. macquarti and specimens of H. illota and H. lasiophthalma exhibited higher infection rates outside of Tompkins County. Two T. sparus milleri specimens collected outside the county were infected.

B. Comparison of Infected and Blooded Tabanids from Different Collecting Sites

Data about infections and blood meals of flies collected at each site are presented in Table XI. The sample from Site II (1973) with the infection rate of 57% represents a select population which is described in detail below (Table XVI). Infection rates depended more on the dates of collection than on the specific geographic location. For example, the samples from Sites I and VI collected early in each summer both exhibited very low infection rates (less than 10%). Infection rates of all samples increased as each summer progressed (Section C).

The presence or absence of cattle and/or deer in proximity to a particular collection site (Materials and Methods, Section I A) could not be directly correlated with infection rates. However, those sites where deer were present but which were at least one mile from the nearest herd of grazing cattle (e.g., Sites II, IV) showed as high or higher infection rates than those sites in close proximity to cattle (e.g., Sites V, VI).

Table X. Tabanid Species Collected[1] Outside of Tompkins County

Site II Species	# Collected 1972	# Collected 1973	# Infected 1972	# Infected 1973	% Infected 1972	% Infected 1973
Atylotus bicolor	2		0		0%	
Chrysops callidus	(+1)		(0)		(0%)	
C. cincticornis	3		1		33%	
C. geminatus	1(+6)		(+2)		0%(33%)	
C. indus	3(+2)		1(+1)		33%(50%)	
C. macquarti	(+3)		(+3)		(100%)	
C. vittatus	(+5)		(+1)		(20%)	
SUB-TOTALS:	9(+17)		2(+7)		22%(41%)	
Hybomitra difficilis	24		9		38%	
H. epistates	8	(+9)(1)	1	(+4)(1)	12%	(44%)
H. illota	3		3		100%	
H. lasiophthalma		(+19)(15)		(+12)(9)		(63%)
H. zonalis	1		1		100%	
SUB-TOTALS:	36	(+28)	14	(+16)	39%	(57%)
Tabanus marginalis	1		0		0%	
T. nigripes	1		0		0%	
T. sparus milleri	2		2		100%	
T. sulcifrons	2		0		0%	
SUB-TOTALS:	6		2		33%	
Chenango Co. Sites						
Atylotus thoracicus	(+5)		(0)		(0%)	
Chrysops geminatus	2(1)		1(1)		50%	
C. shermani	(+1)		(+1)		(100%)	
C. vittatus	1		0		0%	
SUB-TOTALS:	3(+6)		1(+1)		33%(17%)	
TOTALS:	54(+23)		19(+8)		35%(34%)	

[1]Notations concerning flies collected by different methods and number of blooded flies are the same as those in Table VI.

[2]Chenango County Sites are listed under "Miscellaneous Sites" in the Materials and Methods Section (I A).

Table XI. Comparison of Tabanids at Different Collecting Sites

Sites	#Spp.	Dates	#Dissected	#Infected	%Infected		#Blooded	%Blooded Infected	%Blooded Not Inf.
I	(7)	6 Jun - 13 Jun	89	4	4%		1		
II	(16)	21 Jun - 14 Aug	68	25	35%	43%	0	0%	0%
	(2)[2]	25 Jun - 1 Jul	28	16	57%		6(10)[3]	62%	50%
III	(2)	11 Jul - 13 Jul	10	0	0%		0	0%	0%
IVA	(10)	4 Jul - 26 Aug	105	24	23%		12(5)	21%	15%
B	(11)	15 Jul - 26 Aug	50	6	12%	19%	10(2)	33%	23%
V	(19)[2]	5 Jun - 31 Aug	229	89	39%		(9)	10%	0%
VI	(8)[2]	7 Jun - 20 Jun	48	4	8%		0	0%	0%
N.Lansing	(2)	29 Jul	5	2	40%		0	0%	0%
Jam Pond[4]	(1)	21 Jul	4	0	0%		0	0%	0%
Mud Pond[4]	(4)	4 Aug	5	2	40%		(2)	100%	0%
			641	172	27%		28(28)	16%	6%

[1]Benzidine tests were not performed on this group of flies.

[2]1973 collections; others are 1972.

[3]The number of blooded flies in parentheses refer to infected flies only.

[4]Chenango County.

The percentage of infected flies which were blooded was higher than that of the non-infected group. This relationship was true for all sites (Table XI). The association of blood feeding and infection is discussed in Section D.

C. Seasonal Occurrence of Trypanosome Infections in Tabanids

The rates of infection of tabanids calculated on a biweekly basis for each summer are presented in Table XII. Only tabanids collected in Tompkins County are included in the table. The infection rates of the most abundant flies collected calculated on a weekly basis are presented in Table XIII. Weekly samples which comprised five or more specimens are included in the table. Both tables (XII and XIII) indicate that the infection of a given species depends on the part of the summer in which it was collected rather than on a species-specific susceptibility. This pattern of infection is similar to infections which increase during the late summer and early fall characteristic of some insect-borne microorganisms which multiply in vertebrate reservoir hosts during the season when the vectors are active, e.g., the equine encephalitides.

Regular tabanid collections were not made at sites outside of Tompkins County. However, a few isolated samples from Schuyler County (Site II) are presented below with their infection rates:

1972	# Collected	%Infected
June 21	35	42%
July 19, 20, 29	24	33%
August 14	10	30%

Table XII. Seasonal Occurrence of Infections in Female Tabanids (Tompkins County)

Dates	1972		1973	
	# Collected	% Infected	# Collected	% Infected
June 1-15	95	4%	62	10%
16-30	--*		49	18%
July 1-15	43	6%	35	14%
16-31	69	18%	75	40%
Aug. 1-15	29	28%	28	57%
16-31	23	22%	28	71%
	259	14%	277	34%

*Heavy rainfall and low temperatures precluded regular sampling during this period.

Table XIII. Weekly Infection Rates of the Most Abundant Tabanid Species Collected

Summer 1972	Percent Infected per Week*											
	1	2	3	4	5	6	7	8	9	10	11	12
H. lasiophthalma	0	2										
T. similis						0	10		15			
T. quinquevittatus						13			35			
T. sulcifrons												33
Summer 1973												
H. lasiophthalma	0		4									
T. similis					9	16	36					
T. quinquevittatus						0	30	45	64			
T. sulcifrons										66	64	80

*Each percentage was calculated using at least five specimens.

The percentage of infection at Site II in June was higher than that observed in the same month in Tompkins County (Table XII). The large number of infected Hybomitra difficilis collected at Site II on the June date accounted for the overall high rate of infection. Twenty-four of the 35 flies collected were this species (Table X). The high infection rate of this species may reflect a species-specific characteristic or the presence of an abundant source of infection. The infection rates in July and August at Site II were comparable to those observed in Tompkins County.

Additional tabanid collections made outside of Tompkins County included a netted sample from Site II in 1973 (Table XVI) and a small number of Chrysops specimens (N=4) collected in Chenango County (Mud Pond, 4 August 1972). The latter flies were 50% infected (Table X).

The lower infection rate among early summer species (Table XIII) indicates that either the late species have a higher susceptibility to infection or, as suggested above, the source of infection available to all species increases as the summer progresses.

D. Blood Meals and Parity

Benzidine Test Results. All references to "blooded" flies refer to flies which reacted positively to benzidine testing. The percentages of blooded flies among those tested are given in Table XIV.

Table XIV. Incidence of Blooded Flies

	# Collected* (# Blooded)		% Blooded		
	1972	1973	1972	1973	Avg.
Canopy Trap Collections	202(30)	255(9)	15%	4%	8%
Insect Net Collections	28(1)	22(0)	4%	0%	2%
TOTALS:	230(31)	277(9)	13%	3%	8%

*The total number of flies collected is not consistent with the figures in Table IV since benzidine testing in 1972 began after 106 flies had been collected.

The low incidence of blooded flies is consistent with the trapping methods used. The canopy trap is designed to attract flies seeking a blood meal. Similarly, most of the flies collected with an insect net were probably searching for a blood meal. Tabanids disturbed during feeding will continue to seek blood from the same host or a different host until they have fed to repletion (James and Harwood, 1969). This intermittent feeding habit may account for the presence of blooded flies in the canopy trap and those collected with a net.

The relation of blood-fed flies to trypanosome-infected flies is presented in Table XV. The incidences of infected flies among the blooded and non-blooded groups collected in the canopy trap were different. Blooded flies were more often infected than non-blooded flies. A blooded fly entering a canopy trap may have either a mammalian trypanosome infection or an insect trypanosomatid flagellate infection. The former type of infection is acquired by imbibition of infected blood (direct transmission) and the latter by contact with infective material on the surface of a host (indirect transmission).

The insect net collections in Tables XIV and XV do not include a sample of a select population of flies observed swarming near Site II. These flies were swarming over a road in the early morning hours (7:00 to 9:00 A.M.) of 25 June and 1 July 1973 (Pechuman, 1973). Data concerning this group of flies are presented in Table XVI.

Table XV. Relation of Blood Feeding to Infection

Canopy Trap Collections*

Total Flies				Blooded Flies				Non-Blooded Flies			
#(#Infected)		% Infected		#(#Infected)		% Infected		#(#Infected)		% Infected	
1972	1973	1972	1973	1972	1973	1972	1973	1972	1973	1972	1973
202(46)	255(92)	23	36	30(9)	9(9)	30	100	172(37)	246(83)	22	34

Insect Net Collections

28(8)	22(1)	28	4	1(1)	0	100		27(7)	22(1)	26	4

Totals

230(54)	277(93)	23%	34%	31(10)	9(9)	32%	100%	199(44)	268(84)	22%	31%

*Same footnote as for Table XIV.

Table XVI. Swarming Flies Collected with Insect Net (Site II)

Species	# Dissected		# Infected		% Infected
	Females	Males	Females	Males	Females
H. lasiophthalma	19(15[1])	22	12[2](9)	0	63%
H. epistates	9(1)	10	4[3](1)	0	44%
	28(16)	32	16(10)	0	57%

[1]Numbers in parentheses refer to the number of blooded flies (positive benzidine test) in the group preceding the parenthesis.

[2]Some flies were parous (3) and others, gravid (9).

[3]All flies were parous.

The high percentage of blooded female flies (57%) observed (Table XVI) may be related to their unusual swarming behavior. There was a high infection rate of blooded flies (62%) as well as total flies (57%) and all infected flies were gravid or parous. If H. lasiophthalma is anautogenous, as suggested by Thomas (1972), the finding of only gravid or parous infected flies implies that these insects have all had a blood meal. These facts do not provide evidence for differentiating solely insectan trypanosomatid infections (acquired by contamination) from mammalian trypanosome infections (acquired by blood-feeding) since they are consistent with both types of infections. Swarming and longevity (as evidenced by high parous rates) provide opportunities for acquisition of an infection transmitted by contamination with infected flies or their fecal matter. Blood-feeding may provide a further opportunity to acquire such an infection if more than one fly is present on a host or if other flies have contaminated the host's surface with infective fecal material. The act of blood-feeding provides an opportunity to acquire infections with blood-borne mammalian trypanosomes.

The midguts of two blooded specimens of H. lasiophthalma, among those swarming at Site II (Table XVI), contained distinctive erythrocytes identical to the crenulated, sickle-shaped red cells seen in heparinized deer blood.

Parity Determinations. Parity data for the four most abundant tabanid species collected in 1973 are presented in Tables XVII (H. lasiophthalma), XVIII (T. similis) XIX (T.

Table XVII. Parity Determinations - Hybomitra lasiophthalma

1973 (Tompkins County)

	# Fly Specimens			
	# Dissected	w/Distended Pedicels	w/Dilatations	%Parous (by week)
June				
19	1			
				14%
20	6		1	
25	4	1	3	100%
July				
2	2		2	100%
18	1			0%
	14	1	6	50%

Table XVIII. Parity Determinations - Tabanus similis

1973 (Tompkins County)

	# Fly Specimens				
	#Dissected	w/Distended Pedicels	w/Dilatations	%Parous (by week)	w/Retained Oöcytes
June					
19	5			0%	
20	2				
25	2	1		50%	
July					
2	8		4*	45%	1*
5	3		1		
10	6	1	2	50%	
16	10	1	6*	73%	1*
18	1	1			
26	3	1	2*	100%	2*
Aug.					
13	1		1*	100%	1*
	41	5	16	51%	5

*The asterisk indicates the group on the same line from which retained oöcyte observation(s) was made.

Table XIX. Parity Determinations - Tabanus quinquevittatus

1973 (Tompkins County)

	# Fly Specimens				
	#Dissected	w/Distended Pedicels	w/Dilatations	%Parous (by week)	w/Retained Oöcytes
July					
2	1				
5	3		1	25%	
10	6		1	17%	
16	2		2		
18	11	2*	1	38%	1*
23	6	1			
26	16	8*	2	50%	1*
30	10	5*	2		1*
Aug.				64%	
2	4	1	1*		1*
8	3	1	1	67%	
13	1	1			
16	3	1	2	100%	
24	2	2		100%	1
	68	22	13	51%	5

* Same footnote as on Table XVIII.

quinquevittatus) and XX (T. sulcifrons). Parity data were not determined for flies collected before June 19; therefore, H. lasiophthalma, which is an early summer species, is represented by only a small sample of flies in Table XVII.

The number of nulliparous flies was relatively high near the beginning of the flight season for each species and gradually diminished. This finding suggests that these species are anautogenous (Thomas, 1972). The one nulliparous specimen of H. lasiophthalma collected near the end of this species's flight season was in perfect condition, indicating that it had probably recently emerged.

The repeated collection of nulliparous flies of a given species in a Manitoba canopy trap may indicate that the species is anautogenous (Thomas, 1972). Thomas, using this criterion, regarded H. lasiophthalma as anautogenous. In addition to the abundant species mentioned above, three other species included nulliparous individuals:

	Nulliparous	Parous
Chrysops callidus	4	5
Hybomitra epistates	5	4
Tabanus sparus milleri	1	5

Nulliparous specimens of species represented by fewer than 5 flies in the collections are not considered here. The parity data suggest that C. callidus, H. epistates, H. lasiophthalma, T. quinquevittatus, T. similis, and T. sulcifrons are anautogenous species.

One T. marginalis, which emerged in the laboratory from Sphagnum moss and fed on a sucrose-water solution (Section I E)

Table XX. Parity Determinations - Tabanus sulcifrons

1973 (Tompkins County)

	# Fly Specimens				
	#Dissected	w/Distended Pedicels	w/Dilatations	%Parous (by week)	w/Retained Oöcytes
July 26	3			0%	
30	3				
Aug. 2	1		1	25%	
8	6	1	3	67%	
13	6		4		
16	11	2	8*	82%	1*
24	5	3	1	80%	
28	2	2*			1*
30	1	1		100%	
31	1	1			
	39	10	17	69%	2

*Same footnote as on Table XVIII.

for 48-72 hours, exhibited fully-developed ova when dissected. In addition, two flies, one unfed specimen of Atylotus bicolor raised from a larva (Section I E) and one unfed C. frigidus which emerged from Sphagum moss in the laboratory, exhibited fully-developed ova within one week post-emergence. These observations suggest that T. marginalis, A. bicolor and C. frigidus are autogenous at least during the first gonotrophic cycle.

Parity Data in Relation to Infection. Parity determinations were made on a selected number of infected flies (12 specimens) collected in 1972 and the majority of flies (220 specimens) collected in 1973. All 12 infected flies from 1972 and all of those in the group from 1973 which were infected with flagellates were parous, gravid or blooded. The specific data regarding parity and blood meals of these infected flies are presented in Table XXI.

The individual nulliparous infected fly, a T. quinquevittatus (Table XXI), was cultured to learn more about the nature of the infection. This specimen was one of two which produced pure choanomastigote (Crithidia sp.) cultures (Section III A). Therefore, the infection from the nulliparous T. quinquevittatus can be excluded from those being considered as possible mammalian trypanosome infections.

The modes of egg development (autogeny vs. anautogeny) are not known for most of the species in this study (Table IV). Some evidence for anautogenous development of the four species which represented the most abundant flies and the majority of infected flies in this study (Table VI) has been presented.

Table XXI. Parity Determinations of Infected Flies

	# Infected[1]	# Nulliparous	# Gravid	# Parous
1972	12	0	1[2]	11
1973	85	1[2]	0	84
Swarming at Site II	16		9[3]	7
	113	1	10	102

[1]Infected flies are only those infected flies on which parity determinations were made.

[2]These flies were blooded.

[3]Eight flies were blooded.

Therefore, if anautogenous development occurs in these species, all infected flies of these species could have acquired flagellate infections from the imbibition of infected blood or by contamination with infective material (from other flies) deposited on the host's surface.

As in the case with blood determinations, the parity data do not provide sufficient information to specifically identify the types of infections occurring in the tabanids. However, the finding that all flies infected with non-choanomastigote flagellates are either gravid or parous suggests that these flies became infected only after the amount of time necessary to mature ova had elapsed. During that time, anautogenous species would have ingested a blood meal.

E. Freshly-Emerged Tabanids

Tabanids Raised from Larvae. Dissection data for tabanids raised from larvae in the laboratory are given in Table XXII. In addition, one female C. univittatus and one male C. callidus were raised from larvae collected at Site I in 1972. Both were negative for flagellates. The specimen of A. bicolor (Table XXII) is discussed as an example of autogenous development in Section I D. The absence of infected flies among all tabanids raised from larvae indicates that flagellate infections are acquired after emergence from the pupae.

Tabanids Caught in Emergence Traps. Dissection data for tabanids trapped in emergence cages are given in Table XXIII.

Table XXII. Flies Raised from Larvae (1973)

Site	Sex	Species	#	Date of Larvae Collection	#Infected	Parity
V	F	C.callidus	3	24 May	0	Nulliparous
			1	6 June	0	Nulliparous
		C.indus	3	24 May	0	Nulliparous
		C.univittatus	3	24 May	0	Nulliparous
VI		A.bicolor	(1)	7 June	*	Gravid
TOTAL(F):			10		0	
V	M	C.callidus	2	5 June	0	
		C.indus	1	24 May	0	
		C.univittatus	1	24 May	0	
			3	6 June	0	
			2	14 June	0	
		T.reinwardtii	1	12 June	0	
TOTAL(M):			10		0	

*not examined

Table XIII. Flies Caught in Emergence Traps (1973)

Site	Sex	Species	#	Date Collected	#Infected	Parity
V	F	C.callidus	2	14 June	0	Nulliparous
			2	25 June	0	Nulliparous
		C.indus	10	12 June	0	Nulliparous
			2	14 June	0	Nulliparous
		C.univittatus	1	25 June	0	Nulliparous
		C.vittatus	1	14 June	0	Nulliparous
TOTAL(F):			18		0	
	M	C.callidus	4	12 June	0	
			3	14 June	0	
			2	19 June	0	
		C.indus	2	7 June	0	
			6	12 June	0	
			1	14 June	0	
		C.sackeni	1	19 June	0	
		C.univittatus	1	19 June	0	
			1	25 June	0	
			1	10 July	0	
		C.vittatus	1	14 June	0	
TOTAL(M):			23		0	

In addition, two T. lineola (one male, one female), one T. marginalis (female) and one C. frigidus (female) emerged from sediment (collected in Tompkins County) in the laboratory.* None were infected with flagellates. The C. frigidus had well-developed ova (gravid) as discussed in Section I D.

None of the flies in Table XXIII harbored flagellates. By comparison, infected flies of some of the same species were collected at the same site (V) in the canopy trap (or insect net). These infected flies are listed below:

1973	Species	Date Collected	#	#Infected
	C. callidus	25 June	5	2
		5 July (netted)	1	1
	C. vittatus	24 August	1	1

The absence of infection of flies caught in the emergence cages is consistent with the results of laboratory rearing of tabanids. Flagellate infections are acquired after flies emerge from the pupal stage.

F. Examination of Male Tabanids

Freshly-emerged male tabanids are listed in Tables XXII and XXIII, and Section E. Some additional male tabanids were trapped in the canopy trap or netted. These are listed in Table XVI (swarming flies at Site II) and below:

*These specimens were kindly provided by Messrs. Gary R. Mullen and Alan Cochrane.

1972	Species	Date Collected	#	#Infected
(netted)	H. lasiophthalma	12 June-Ithaca	1*	0
(netted)	A. thoracicus	21 July-Jam Pond	3	0
1973				
	H. lasiophthalma	14 June-Slaterville	2	0
	T. quinquevittatus	10 July-Site V	2	0
		18 July-Site V	2	0
		23 July-Site V	1	0
			11	0

None of the 78 males dissected (including 35 freshly-emerged specimens) were infected. This finding indicates that flagellate infections are restricted to female tabanids.

II. Flagellate Forms Observed in Tabanids

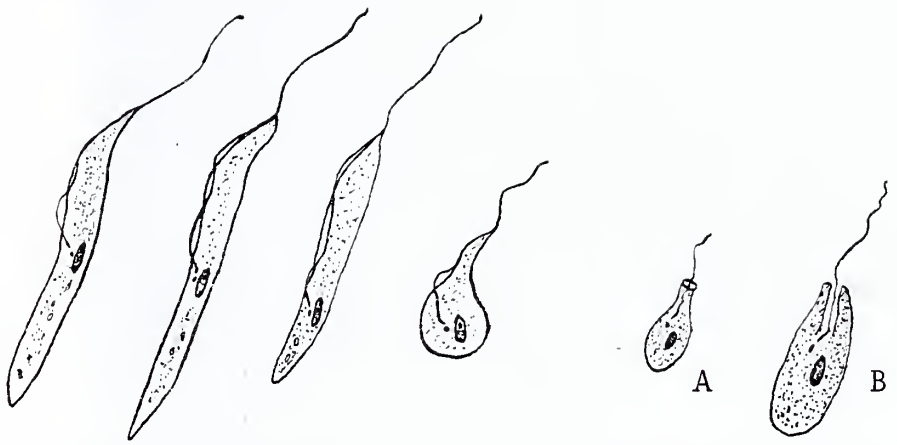
A. Morphological Forms

Motile and immotile forms were seen in the alimentary tracts of tabanids. All forms observed are illustrated in Figure 10. Motile forms were epimastigotes in all but three cases. The latter flies exhibited choanomastigote forms. Immotile forms comprised amastigotes of two types. All of these forms will be discussed below.

Epimastigote Forms. The epimastigotes (Figures 10-13) exhibited a diversity of shapes depending on their location in

*This specimen was kindly provided by John J.S. Burton.

MOTILE FORMS



Epimastigotes

Choanomastigotes

IMMOTILE FORMS

Type I

Type II

"Cystic"

Amastigotes

Figure 10. Developmental Stages of Flagellates Found in Tabanid Intestines. Drawn to scale: 2 mm. ≅ 1 μ.

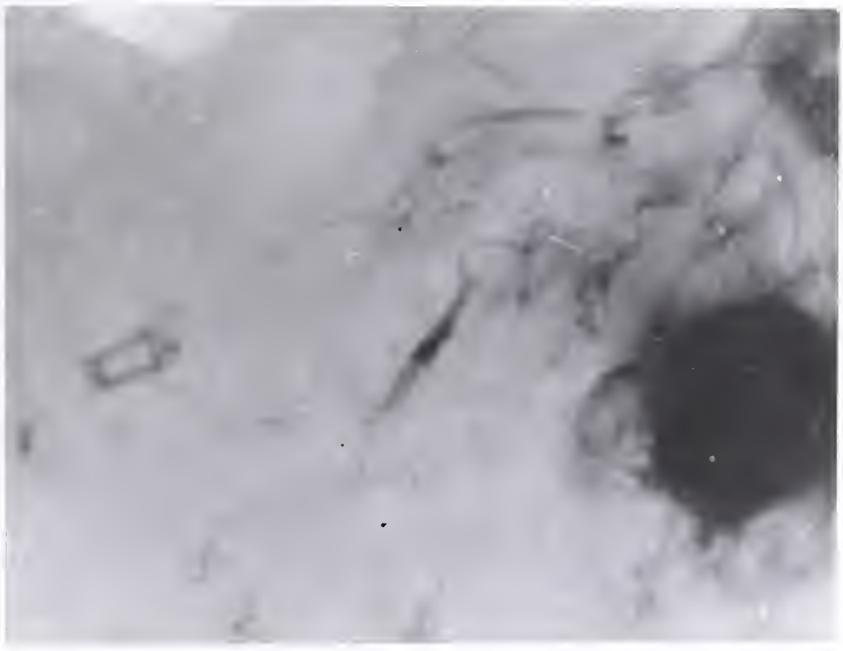


Figure 11. Epimastigote (in foregut of blooded Hybomitra lasiophthalma collected at Site II, 1973). 3000 X.

Figure 12. Transitional epimastigotes (in midgut of blooded H. lasiophthalma collected at Site II, 1973). 3000 X.

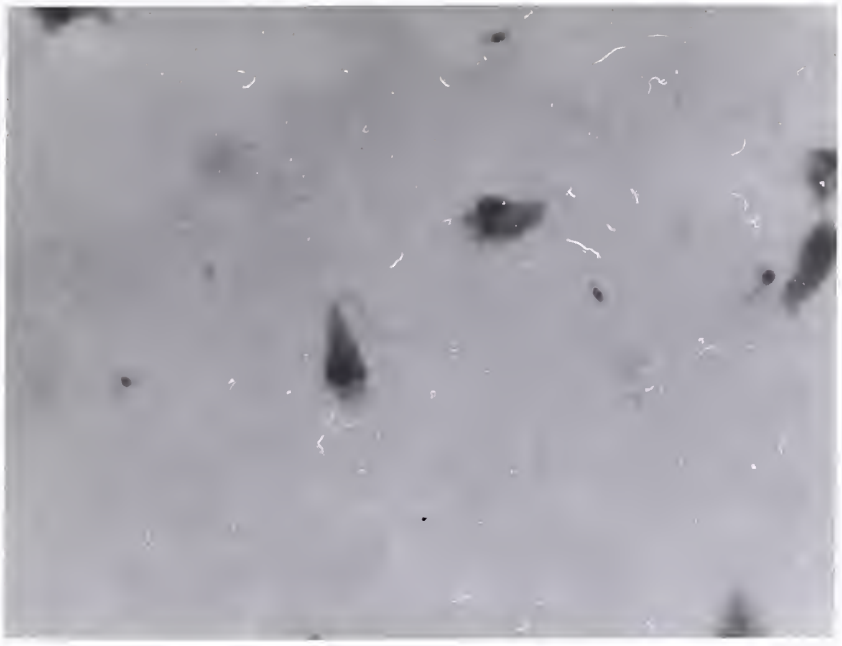


Figure 13. Pyriform epimastigote (in rectum of H. lasiophthalma collected at Site II, 1973). 3000 X.

Figure 14. Transitional epimastigote (left) and (?) sphaeromastigote (in midgut of blooded H. lasiophthalma collected at Site II, 1973). 3000 X.

the fly gut. In general, shorter epimastigotes were found in the distal than in the proximal segments. Their total lengths (L) ranged from about 10 μ to 30 μ. All epimastigote forms had the typical characteristics: a juxtanuclear kinetoplast and a flagellum emerging from the side of the body to run along the body or along a short undulating membrane. A few epimastigotes were observed with the nucleus closer to the posterior end of the cell than the middle (Figures 10, 14). These were similar to a variant of epimastigote forms of T. theileri reported in cultures from a cow (Wells, 1971b). The cytoplasm of all epimastigotes contained granules and vacuoles resembling the cytoplasmic inclusions seen in many Trypanosoma species (Herbert, 1965a). Epimastigotes were observed swimming freely in the gut lumen or massed together by their anterior ends in rosette formations (Figures 15, 16). Epimastigote forms represent Blastocrithidia or Trypanosoma species.

A very few rounded motile forms were observed. These resembled the sphaeromastigote forms considered transitional between mastigote and amastigote forms (Hoare, 1972). One of these forms which could also be a degenerate epimastigote appears on the right side of Figure 14.

Choanomastigote Forms. The choanomastigotes seen in three flies, a C. cincticornis, a H. difficilis and a T. marginalis comprised two basic types. Choanomastigotes in the first two species were very small (8-10 μ = L, 4-6 μ = body length) active "barley corn" and "bullet"-shaped forms. Each organism had a truncate anterior end with a prominent circular rim around

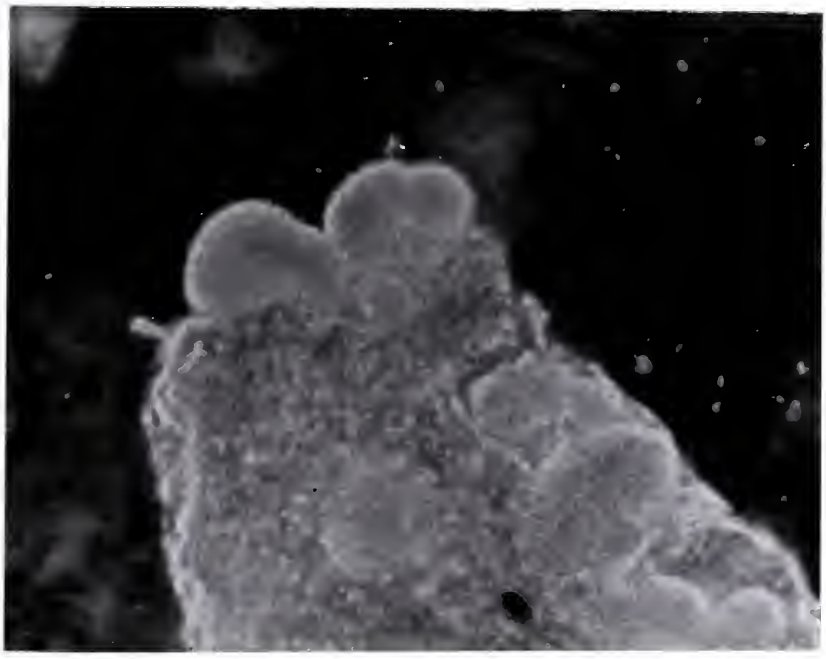


Figure 15. Rosettes of transitional epimastigotes (in anterior hindgut of blooded H. lasiophthalma collected at Site II, 1973). Living preparation. 128 X.

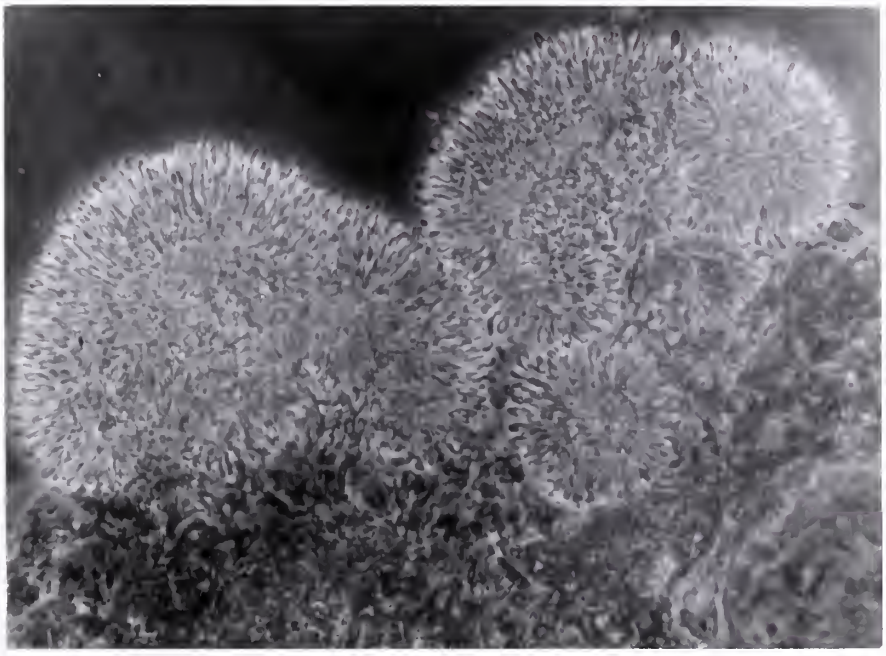


Figure 16. Same as Figure 15. 400 X.

the opening of the reservoir (Figure 10, Type A). The choanomastigotes from T. marginalis were ovoid, larger (15-20 μ = L, 10-12 μ = body length) forms. Each one had a tubular anterior reservoir without a noticeable rim. The reservoir extended nearly to the level of the kinetoplast (Figure 10, Type B). Both types of choanomastigotes had granular cytoplasms. The kinetoplast in both forms was laterally situated and 1/3 to ½ the body length from the anterior end. The choanomastigotes seen in all of the flies were swimming freely in the lumen of the gut. Choanomastigotes are Crithidia species.

Amastigote Forms. The immotile forms observed were amastigotes of two types. The most commonly seen were narrow "barley corn"-shaped forms with a slightly tapered anterior end. These are designated "Type I" forms. Type I amastigotes (Figures 10, 17, 18) were 4-7 μ long and 2-3 μ wide. In living preparations, the cytoplasm was granular. Occasionally a form exhibited a flagellar remnant at the anterior end. In stained preparations, a kinetoplast was observed adjacent and lateral to the nucleus or behind the nucleus in the posterior (slightly wider) end of the cell.

A "vacuolated" form of Type I amastigote was observed in two individual flies (H. illota and H. sodalis). This form was similar to the Type I amastigote but exhibited a large transparent lateral vacuole or a clear shell (Figure 10). This cyst-like form may represent a resting stage or a degenerate form. Cyst-like forms were found on both occasions in conjunction with typical Type I amastigotes. It is possible that these

Figure 17. Type I amastigotes (embedded in hindgut of H. lasiophthalma collected at Site VI, 1973). 1350 X.

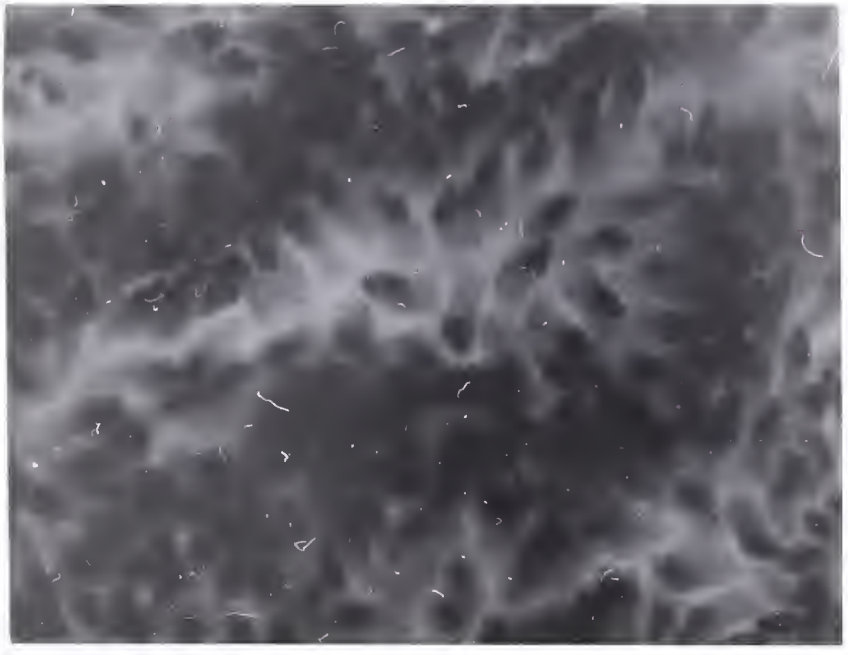


Figure 18. Same as Figure 17. 3000 X.

unusual forms represent a stage in the life-cycle of a flagellate parasite other than the one of which Type I amastigotes are a part.

Type II amastigotes (Figures 10, 19) were larger (6-10 μ by 3-5 μ) and more granular in appearance than the Type I forms. Each Type II form was "pear"-shaped with a wide posterior end and often had a flagellar remnant emerging from a rudimentary tubular anterior reservoir. The kinetoplast was poorly visualized in a juxtanuclear position. With the exceptions of the lack of a flagellum and the immobility, the Type II amastigote closely resembled the choanomastigote form seen in T. marginalis. Both Type I and II amastigotes were found free in the gut lumen or embedded with their anterior ends lodged in the intestinal wall (Figures 18-21).

B. Occurrence of Forms in Fly Samples

The percentages of tabanids infected with motile and/or immotile forms are listed in Table XXIV. Only three flies harbored motile choanomastigote forms. One of these was a pure choanomastigote infection (T. marginalis). The other two choanomastigote infections (C. cincticornis and H. difficilis) were mixed infections with epimastigote forms. All flies infected with immotile forms exhibited Type I amastigote forms. A small percentage of these flies (14%) also harbored Type II amastigotes. Thus, Type I and Type II forms were always found together as mixed infections.

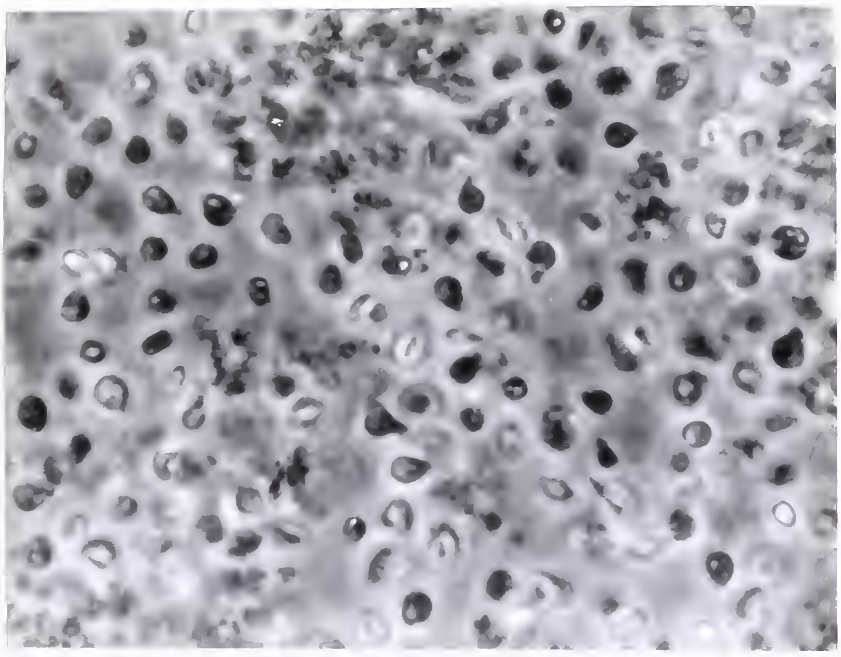


Figure 19. Type I and II amastigotes (in hindgut of Tabanus sulcifrons collected at Site V, 1973). Living preparation. 900 X.

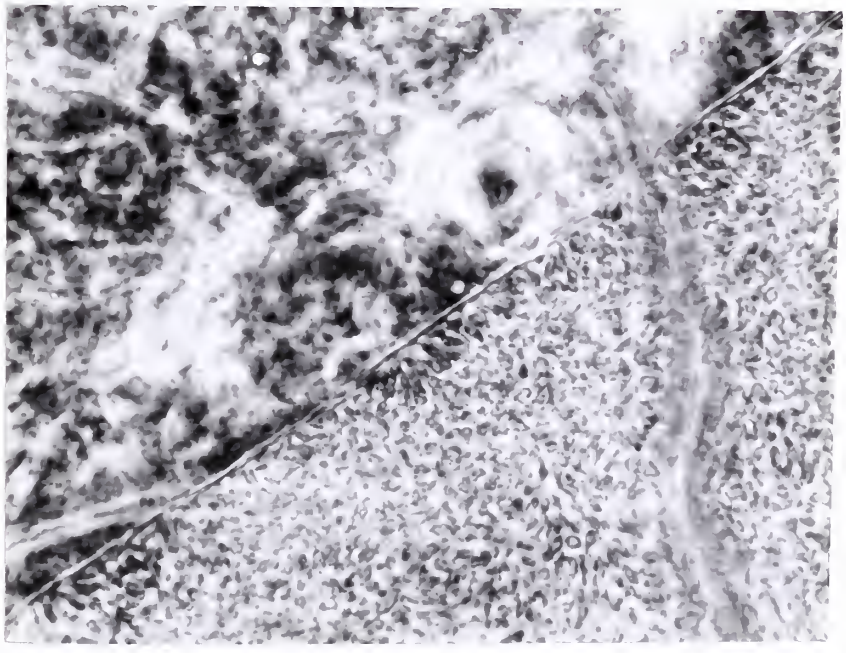


Figure 20. Type I and II amastigotes (in hindgut of T. sulcifrons collected at Site IV A, 1973). The intima appears as an oblique line across the picture. Flagellates are seen embedded in the membrane and also free in the gut lumen. Living preparation. 400 X.

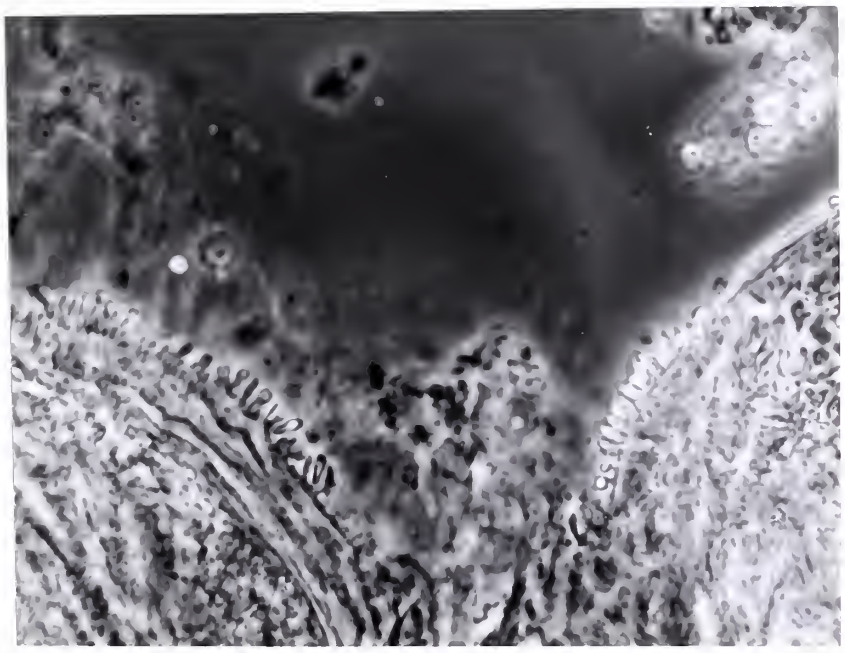


Figure 21. Type I amastigotes (embedded in intima of rectum of Tabanus marginalis collected at Site V, 1973). Living preparation. 640 X.

Table XXIV. Incidence of Motile and Immotile Forms in Tabanids

	# Infected	% Motile Forms		% Immotile Forms		
		Epimastigote	Choanomastigote	Type I	Type II	"cyst"
1972	63	36%	3%	88%	8%	1%
1973	109	39%	1%	97%	16%	1%
	172	32%	2%	94%	13%	1%

Table XXIV indicates that about the same number of tabanids were infected with epimastigote, choanomastigote and Type I amastigote forms in 1972 as in 1973. The larger sample size and more thorough examination of infections found in 1973 may explain the higher percentage with Type II amastigotes found that year.

The percentages of flies infected with single flagellate forms and combinations of motile and immotile forms are given in Table XXV. Since motile forms are much easier to find than immotile forms, the higher percentage of flies having pure infections with motile forms in 1972 may again be explained by the larger and more thorough study in 1973. Many of the flies having mixed infections in 1973 harbored a very small number of immotile forms which were found only after extensive searching. The percentages for 1972 do not equal 100% because no flagellates were observed in one fly (H. zonalis) which was found to be infected only after the intestine was cultured. It can be concluded from Table XXV that immotile forms were the predominant flagellate forms seen in infected flies. The majority of the infected flies had pure infections with immotile forms.

C. Distribution of Forms in Each Fly

The different morphological forms of trypanosomatid parasites were grouped according to their anatomic locations.

Salivary Glands and Crop. Salivary glands from most of the

Table XXV. Incidence of Flies with Single and Multiple Forms

	#Infected	% Motile Forms Only	% Immotile Forms Only	%Motile and Immotile Forms
1972	63	12%	62%	25%
1973	109	2%	60%	38%
	172	5%	60%	33%

flies harboring intestinal flagellates in 1973 were successfully removed and examined (93/109). The salivary glands from all species of infected flies except C. ater and C. mitis were examined. In addition, salivary glands from 25 uninfected H. lasiophthalma were examined. No flagellates were observed in any of the salivary gland preparations (N=118).

The pharyngeal duct leading to the crop was examined in all specimens on which salivary gland dissections were done. In a small number of these cases, a dilated crop was found and examined. No flagellates were seen in the crop or associated duct of these flies.

The above findings indicate that the flagellates observed in this study do not inhabit the salivary glands or pharyngeal portion of the alimentary tract. Therefore, it is very unlikely that these flagellates are transmitted by the salivarian (anterior) route.

Alimentary Tract. All of the flagellates were observed in the alimentary tract below the level of the pharynx. The anatomic locations of different forms will be discussed.

Motile Forms. Data regarding the localization of different forms in those flies harboring motile flagellates are presented in Table XXVI. Examination of the table reveals that elongate epimastigote forms were most often located in the midgut (MG) (Figures 12, 14) and anterior hindgut (HG). In the latter location, they were often amassed in rosette formations (Figures 15, 16). Rosettes occurring in the MG and HG indicated actively dividing flagellates. A few flies exhibited

Table XXVI. Location of Flagellate Forms in Tabanids Harboring Motile Forms

Species*	Motile Forms			Immotile Forms	
	Epimastigotes (elongate)	Epimastigotes (short)	Choano-mastigotes	Amastigotes Type I	Amastigotes Type II
1972					
C. cincticornis		G	G		
C. geminatus (2)		G		G	
" (B)		MG, G		G	
C. macquarti		G		G	
"		MG, G		G	
"	G(R)	G		G	
C. vittatus(2)		G		G	
H. difficilis	MG, G	G		G	
"		G		G	G
"		G	G		
"	G(R)	G			
" (3)		G			
"		G		G	
H. epistates		G			
H. illota		MG, G		G	
H. lasiophthalma	G	G		G	G
T. calens		MG, G		G	
T. quinquevittatus		MG, G		G	
" (B)	MG, G	MG, G		G	

*Individual specimens are listed unless a number of specimens appears in parenthesis following the species name.

Table XXVI, Continued

Species	Motile Forms: Epimastigotes (elongate)	Motile Forms: Epimastigotes (short)	Motile Forms: Choanomastigotes	Immotile Forms: Amastigotes Type I	Immotile Forms: Amastigotes Type II
1973					
C. ater		P-HG,Rm		P-HG,Rm	P-HG,Rm
C. callidus		HG		HG	
C. mitis	FG,MG,HG,Rm	MG,HG,Rm		MG,HG	HG,Rm
C. vittatus	HG			HG,Rm	HG,Rm
H. epistates (S)		HG,Rm		HG,Rm	
" (S)	A-HG	HG,Rm		P-HG,Rm	
H. lasiophthalma (S)	MG,HG(R),Rm	MG,HG,Rm		MG,HG,Rm	
" (S)	MG,HG(R),Rm	MG,HG,Rm		HG,Rm	
" (B,S)	MG,HG	HG,Rm		HG	
" (B,S)	FG,MG,HG,Rm	MG,HG,Rm			
" (S)	FG,MG	MG		HG,Rm	
" (B,S)	MG,A-HG(R)	HG		P-HG,Rm	
" (5) (B,S)	FG,MG,HG	HG,Rm		HG,Rm	
"	MG,HG	HG			
"	HG,Rm	HG,Rm		HG,Rm	
H. typhus		HG,Rm		MG,P-HG,Rm	
T. calens	MG,A-HG(R)	A-HG		P-HG,Rm	
T. marginalis			Rm		
"	HG,Rm	HG,Rm		MG,HG,Rm	
T. quinquevittatus	HG	HG		HG,Rm	
"	MG,HG,Rm	MG,HG,Rm		HG,Rm	
"		P-HG,Rm		P-HG,Rm	
"	A-HG(R)			MG,P-HG,RM	
"	HG,Rm	HG,Rm		HG,Rm	
"	HG,Rm	HG,Rm		MG,P-HG,Rm	

Table XXVI, Continued

Species	Motile Forms: Epimastigotes (elongate)	Motile Forms: Epimastigotes (short)	Motile Forms: Choano-Mastigotes	Immotile Forms: Amastigotes Type I	Immotile Forms: Amastigotes Type II
T. quinquevittatus		Rm		Rm	
"		HG, Rm		P-HG, Rm	
" (B)	A-HG(R)			MG, HG	HG
"		Rm		P-HG, Rm	
"		MG, HG		MG, HG	MG, HG
T. similis		A-HG, Rm		P-HG, Rm	
"		Rm		HG, Rm	
"		HG		HG, Rm	
T. sparus milleri		P-HG, Rm		P-HG, Rm	P-HG, Rm
T. sulcifrons		MG, HG, Rm		MG, HG, Rm	
" (B)		Rm		P-HG, Rm	
"		HG, Rm		HG, Rm	
"	MG(R), A-HG	A-HG		P-HG, Rm	
"		MG, HG, Rm		P-HG, Rm	
"	A-HG(R)			MG, P-HG, Rm	

ABBREVIATIONS: FG = foregut, MG = midgut, HG = hindgut, Rm = rectum, G = HG and/or Rm, A- = anterior, P- = posterior
(R) = rosette formations present
(B) = specimen with positive benzidine test for blood
(S) = specimen caught with insect net from swarm of flies
Underlined segments of intestine are those with the heaviest infection of flagellates in any given fly.

elongate epimastigotes in the foregut (FG) (Figure 11). The short epimastigote forms (Figure 13) were located predominantly in the posterior HG and rectum (Rm). In addition to epimastigotes of different lengths, rounded (?sphaeromastigote) forms were occasionally seen in the gut. These forms may represent transitional stages between mastigotes and amastigotes.

Epimastigote forms in the tabanids represent developmental stages in the life history of a Trypanosoma species or a Blastocrithidia species. Morphological criteria are inadequate for separating the epimastigote stages of these two genera.

Choanomastigote forms were found in the posterior intestinal segments (HG and Rm) of the three flies in which they occurred. One of the flies (T. marginalis) had a pure infection of choanomastigotes (Figure 10, Type B). Choanomastigote forms represent the last developmental stage in the life-cycle of Crithidia species. These species are solely insect parasites.

Immotile Forms. Immotile forms were found predominantly in the HG and/or Rm. In 1972, the HG was not separated from the Rm during dissection. Among those flies harboring immotile forms in 1972 (N=56), 91% exhibited these forms in the HG and/or Rm, and 9% had them in the MG as well as one of the other sections. In 1973, analysis of all flies harboring immotile forms (N=108) revealed that 65% had these forms in the HG and Rm, 27% in the HG alone, 6% in the MG as well as HG or Rm and 2% in the Rm alone. When the 1973 percentages for all HG and/or Rm infections are combined as they were in 1972, a comparison of percentages for the two years reveals that the

distribution of immotile forms was identical.

In 1973, the HG was more heavily infected than the Rm in the flies with infections of these two sites. Immotile forms in the MG were always found in small numbers. The MG forms were free in the lumen of the gut and most likely represent forms which floated into the MG from more distal intestinal segments as the alimentary tract was manipulated during dissection.

The distribution of immotile forms occurring in those flies with concomitant infections with motile forms (Table XXVI) was typical of the pattern found in all flies harboring immotile forms. Three flies (two H. lasiophthalma and one T. quinquevittatus) harbored Type I amastigotes oriented in rosette formations in the rectum. The abundance of Type I amastigotes suggests that these forms are capable of extensive multiplication. The three flies with rosette formations provided direct evidence that reproduction by amastigote forms is possible.

The immotile amastigote forms observed in tabanids (Figures 10, 17-21) may represent early developmental stages of either Crithidia, Blastocrithidia or Trypanosoma species (Figure 2).

III. Cultures Derived from Tabanid Flagellate Forms

The flagellate forms cultured from tabanids provided some information for grouping the fly forms into possible develop-

mental sequences. No flagellates were ever observed in fresh or stained blood smears taken from rabbits bled for culture media preparation. All uninoculated culture tubes (N.N.N., McCoy's, CuF_{10}) incubated with each group of inoculated tubes were negative for flagellates.

N.N.N. Medium (28°). In 1972, segments of gut infected with flagellates were inoculated into N.N.N. medium and cultured at 28°. Positive cultures were maintained by transfer every two weeks.

One-half of the flies (23/47) providing tissue for culture initiation harbored pure infections of Type I amastigotes. The other half harbored the following combinations of morphological forms (the number of flies infected with each combination is given over the total number of flies infected): (1) Type I and II amastigotes (2/47), (2) Type I and II amastigotes and short epimastigotes (2/47), (3) Type I amastigotes and short epimastigotes (9/47), (4) Type I amastigotes and short and long epimastigotes (4/47) (5) short epimastigotes only (3/47), (6) short and long epimastigotes (1/47) (7) short epimastigotes and choanomastigoes (2/47) and (8) Type I and II amastigotes and short and long epimastigotes (1/47). Epimastigotes were found in all incubated cultures. The species from which tissues were taken and the combinations of flagellate forms found in each of these species are listed in Table XXVII.

Type I amastigotes alone or in combination with other forms were found in the tissues of all infected species but two, C. cincticornis and H. zonalis. The former harbored

Table XXVII. Culture Results from Infected Species (1972)

N.N.N. 28°

Species	Flagellate Forms in Fly[1]	#Positive/#Total Cultures[2]
Chrysops cincticornis	(7)	1/1
C. geminatus	(3)	1/3
C. indus	A[3]	1/2
C. macquarti	(3)	1/3
C. shermani	A	1/1
C. vittatus	A(1),(3)	3/3
Hybomitra difficilis	(2)-(7)	7/7
H. illota	A	2/2
H. lasiophthalma	(8)	1/1
H. sodalis	A	1/2
H. zonalis	none	1/1
Tabanus quinquevittatus	(1),(3),(4)	10/13
T. sackeni	A	0/1
T. similis	A	3/4
T. sparus milleri	A	1/2
T. sulcifrons	A	1/1
T. superjumentarius	A	1/1
		36/48 = 75%

[1]Numbers in parentheses refer to combinations of flagellate forms listed in the text (Section III, p. 117).

[2]All positive cultures were pure epimastigote cultures.

[3]"A" indicates that at least one specimen of the species harbored a pure infection of Type I amastigote forms.

short epimastigote forms only and the latter, no flagellate forms. A comparison of all of the combinations of flagellate forms observed in 1972 indicates that all of the infected flies had one of the two forms in common, Type I amastigotes and/or short epimastigotes. Since all of the positive cultures exhibited identical epimastigote forms, it is probable that the forms common to all of the infected flies, i.e., Type I amastigotes and/or short epimastigotes, are the precursors of these culture forms. The morphological details of the culture forms are presented in Section III A. The results of the 1973 culture observations are discussed below.

McCoy's Medium (28°). In 1973, infected tabanid tissues were inoculated into McCoy's culture tubes and incubated at 28°. Positive cultures were maintained by transfer every two weeks. About 37% (30/82) of the infected tabanids cultured had pure Type I amastigote infections. The remainder of the infected flies cultured harbored the same combinations of forms as those observed in 1972: (1) Type I and II amastigotes (8/82), (2) Type I and II amastigotes and short epimastigotes (3/82), (3) Type I amastigotes and short epimastigotes (15/82), (4) Type I amastigotes and short and long epimastigotes (20/82), (5) short epimastigotes only (0/82), (6) short and long epimastigotes (2/82), (7) short epimastigotes and choanomastigotes (0/82), and (8) Type I and II amastigotes and short and long epimastigotes (1/82), as well as (9) Type I and II amastigotes and long epimastigotes (2/82), and (10) choanomastigote forms only (1/82). The species from which tissues were taken and the

combination of flagellate forms found in each of these species are listed in Table XXVIII.

As in 1972, all positive epimastigote cultures resulted from tabanid tissues harboring Type I and/or short epimastigote forms. This further supports designation of these forms as precursors of epimastigotes.

Choanomastigote cultures grew in two cases from tissues harboring Type I and II amastigotes and in a third case, from rectal tissue infected with choanomastigotes only. Inoculation of cultures with Type I and II amastigotes in the former two cases resulted in mixed cultures of epimastigotes and choanomastigotes. After 21 days, one of these cultures (from T. quinquevittatus) exhibited only choanomastigote forms (Section III A).

The following observations strongly suggest that Type II amastigotes are precursors of choanomastigote forms: (1) Type II amastigotes have a close resemblance to the choanomastigotes found in a specimen of T. marginalis (Section II A), (2) Type I and II amastigotes alone were found in the tissues of two flies which, when cultured, produced both epimastigote and choanomastigote forms, and (3) Type I amastigotes and/or short epimastigotes resulted in epimastigote cultures in all 1972 and 1973 cultures.

CuF_{10} Medium (37^{o}). An attempt was made to isolate flagellates at 37^{o} and to compare such culture forms with those observed at 28^{o}. Flagellate forms from five species of infected flies were used to inoculate replicate cultures in N.N.N.

Table XXVIII. Culture Results from Infected Species (1973)

McCoy's (28°)

Species	Flagellate Forms in Fly[1]	Epimastigote Cultures[2]	Choanomastigote Cultures[2]
Chrysops ater	A[3],(2)	2/2	
C. callidus	A,(3)	0/2	
C. culux	A	1/1	
C. mitis	(8)	(1/1)	(1/1)[4]
C. vittatus	(9)	1/1	
Hybomitra epistates	(3),(4)	2/3	
H. illota	A	1/2	
H. lasiophthalma	(1),(4),(6)	5/13	
H. typhus	(3)	1/1	
Tabanus calens	A,(4)	2/2	
T. marginalis	(4),(10)	0/2	1/2[5]
T. quinquevittatus	A,(1)(2)-(4),(9)	17/25	1/25[4,6]
T. similis	A,(3)	4/7	
T. sparus milleri	A,(2)	2/4	
T. sulcifrons	A,(1),(3)(4)	11/15	
T. superjumentarius	A	1/1	
		51/82 = 62%	

[1]Numbers in parentheses refer to combinations of flagellate forms listed in the text (Section III, p. 119).

[2]Number of positive cultures are given over the total number of cultures initiated.

[3]"A" indicates that at least one specimen of the species harbored a pure infection of Type I amastigote forms.

[4]These cultures exhibited epimastigote and choanomastigote forms. (The culture from *T. quinquevittatus* was a mixed culture and at 21 days exhibited only choanomastigote forms.)

[5]This culture resulted from the combination of forms designated (10).

[6]This culture resulted from the combination of forms designated (1).

medium at 28° and CuF_{10} medium at 37°. The tabanid intestinal flagellate forms which were inoculated and the forms observed in each set of cultures are listed in Table XXIX. All control cultures (N.N.N. 28°) showed good growth of epimastigote forms and remained positive for at least two weeks without being transferred. The four positive cultures at 37° exhibited very few flagellates and were unable to support flagellate forms for two weeks. Two of these cultures (T. quinquevittatus and T. superjumentarius) exhibited trypomastigote forms characteristic of the genus Trypanosoma. The observation of trypomastigote forms among tabanid flagellates isolated at 37° indicates that Type I amastigotes probably are stages in the life-cycle of a Trypanosoma species. The morphology of these trypomastigote forms is described in Section III A.

Effects of Media and Temperature Changes on Flagellate Forms. Tabanid flagellates isolated in N.N.N. and McCoy's media at 28° were maintained with transfers at least every two weeks for as long as 18 months. All of the flagellates originally isolated in N.N.N. medium at 28° were successfully transferred to McCoy's medium at 28° and maintained for over 9 months with transfers every two weeks.

Type I amastigotes and short epimastigotes from a T. quinquevittatus specimen were used to initiate a culture in N.N.N. medium (28°) from which an epimastigote was cloned. After 4 months, the clone was transferred to McCoy's medium (28°). Two weeks after transfer, small numbers of trypomastigotes appeared in the culture. The N.N.N. (28°) culture had always exhibited

Table XXIX. Flagellate Forms in CuF_{10} Medium (37°)

Species	Forms Inoculated*	Isolation Medium CuF_{10} 37°	Control Medium N.N.N. 28°
C. macquarti	AI and sE	Negative	Epimastigotes
H. sodalis	AI	Negative	Epimastigotes
T. quinquevittatus	AI	Epimastigotes	Epimastigotes
T. quinquevittatus	AI	Epimastigotes	Epimastigotes
T. quinquevittatus	AI	Epimastigotes and Trypomastigotes	Epimastigotes
T. similis	AI	Negative	Epimastigotes
T. superjumentarius	AI	Epimastigotes and Trypomastigotes	Epimastigotes

*The forms observed in each tabanid which were inoculated into culture media are abbreviated as sE = short epimastigotes, AI = Type I amastigotes.

abundant epimastigotes but no trypomastigotes had ever been observed, possibly indicating that the latter medium provides certain nutrients essential for the growth of trypomastigote forms. Furthermore, as in the two cultures (CuF_{10} 37°) mentioned earlier, the appearance of trypomastigotes indicates that at least one of the tabanid intestinal forms (Type I amastigotes) represents a life stage in the development of a Trypanosoma species.

Established N.N.N. cultures (28°) were transferred to other culture media at 28° and 37° to try to elicit transformations from epimastigote to trypomastigote forms. The results of these transfers are presented in Table XXX. No transformations from one flagellate form to another were observed.

Tabanid flagellate cultures could not be maintained at 37° as long as those at 28°. Epimastigote cultures grown at 37° exhibited large numbers of aberrant and degenerate forms. No multiplication was observed in the 37° cultures. The effect of increased temperature on survival of tabanid flagellates was demonstrated by inoculating duplicate N.N.N. and McCoy's culture tubes at 28° and 37° with epimastigote cultures which had been maintained by transfer every two weeks in N.N.N. at 28° for at least 9 months. The test cultures were examined every two days for a total of 33 days. The survival of flagellates in each of the cultures is listed at the top of p. 126. The relatively poor survival of flagellates from T. quinquevittatus probably resulted from the effects of large numbers of bacteria which were observed in those cultures. With the

Table XXX. Transfer of Flagellates from 28° to 37°

Species	Forms Inoculated[1]	Isolation Medium (N.N.N. 28°)	Transfer Day	Secondary Medium (N.N.N. 37°)
C. cincticornis	C,sE	Epimastigotes	18	Epimastigotes
H. difficilis[2]	sE	Epimastigotes	6	Epimastigotes
H. difficilis	sE	Epimastigotes	14	Epimastigotes
		(N.N.N. 28°)		(CuF_{10} 37°)
H. difficilis	sE	Epimastigotes	14	Epimastigotes
C. indus	AI	Epimastigotes	102	Epimastigotes

[1]Abbreviations for flagellate forms seen in the tabanids and used for inoculation are C = choanomastigotes, sE = short epimastigotes, AI = Type I amastigotes.

[2]The same fly was used for each test with this species.

Original Culture N.N.N. 28°	Survival in Days			
	N.N.N. 28°	McCoy's 28°	N.N.N. 37°	McCoy's 37°
C.cincticornis	33	33	14	14
H.difficilis	33	33	17	17
T.superjumentarius	29	29	17	11
T.quinquevittatus	24	10	5	5
Average	30	26	13	12

exception of the cultures from T. quinquevittatus, which exhibited aberrant forms, all cultures at 28° harbored dividing epimastigote forms often seen in rosette formations (Section III C). No trypomastigote forms were observed in any cultures.

Summary of Cultures Initiated with Tabanid Flagellates. The tabanid flagellate forms used to initiate cultures and the forms which grew in each of these cultures are tabulated in Table XXXI. Those cases identified by species names represent individual observations which were particularly useful for the generic identification of the tabanid flagellates. A comparison of fly forms with culture forms suggested that Type I amastigotes are the precursors of epimastigotes and trypomastigotes and Type II amastigotes are the precursors of choanomastigotes.

Detailed morphological studies (below) of the culture forms provided information useful in the generic identification of the flagellate forms found in tabanids.

Table XXXI. Summary of Cultures Initiated with Tabanid Flagellates

Form(s) Inoculated into Medium	Form(s) Observed in Culture
Motile Forms	
short epimastigotes	epimastigotes
long epimastigotes	epimastigotes
choanomastigotes (*T. marginalis*)	choanomastigotes
Immotile Forms	
Type I amastigotes	epimastigotes
Type I amastigotes (*T. quinquevittatus*)	epimastigotes and trypomastigotes
Type I amastigotes (*T. superjumentarius*)	epimastigotes and trypomastigotes
Type I amastigotes (and short epimastigotes from *T. quinquevittatus* clone)	epimastigotes and trypomastigotes
Type I and II amastigotes	epimastigotes
Type I and II amastigotes (*C. mitis*)	choanomastigotes
Type I and II amastigotes (*T. quinquevittatus*)	choanomastigotes and epimastigotes

A. Morphology of Flagellate Forms in Culture

Flagellate forms observed in cultures included epimastigotes (diverse types), trypomastigotes and choanomastigotes. Morphological examinations of flagellate forms from all cultures (Table IV) were performed using fresh and fixed preparations. Four clone cultures, two prepared from T. quinquevittatus and two from T. superjumentarius, were carefully studied and used for comparison with non-clone cultures. In this way, a culture exhibiting diverse forms arising from the transformation of a single form could be differentiated from a culture having a mixed infection of unrelated flagellate forms.

Epimastigote Forms. (1) Qualitative Observations. A diversity of epimastigote forms were observed in all cultures having epimastigotes. All epimastigotes conformed to the definition of this stage (Figure 1) with regard to the position of the kinetoplast and flagellum. The flagellum emerged from the side of the cell (Figure 22) and ran along the border of the cell. A short, narrow, undulating membrane was apparent near the anterior end of most specimens. Epimastigotes of various shapes were observed. The most common types seen are illustrated in Figure 23.

Older cultures exhibited greater diversity than younger ones. All epimastigote forms appeared to be derived from a typical form. Repeated observations of clone cultures and freshly transferred non-clone cultures indicated that all cultures followed a similar pattern of development. From 1-3 weeks

Figure 22. Epimastigote (tabanid clone culture, N.N.N. 28°). 3000 X.

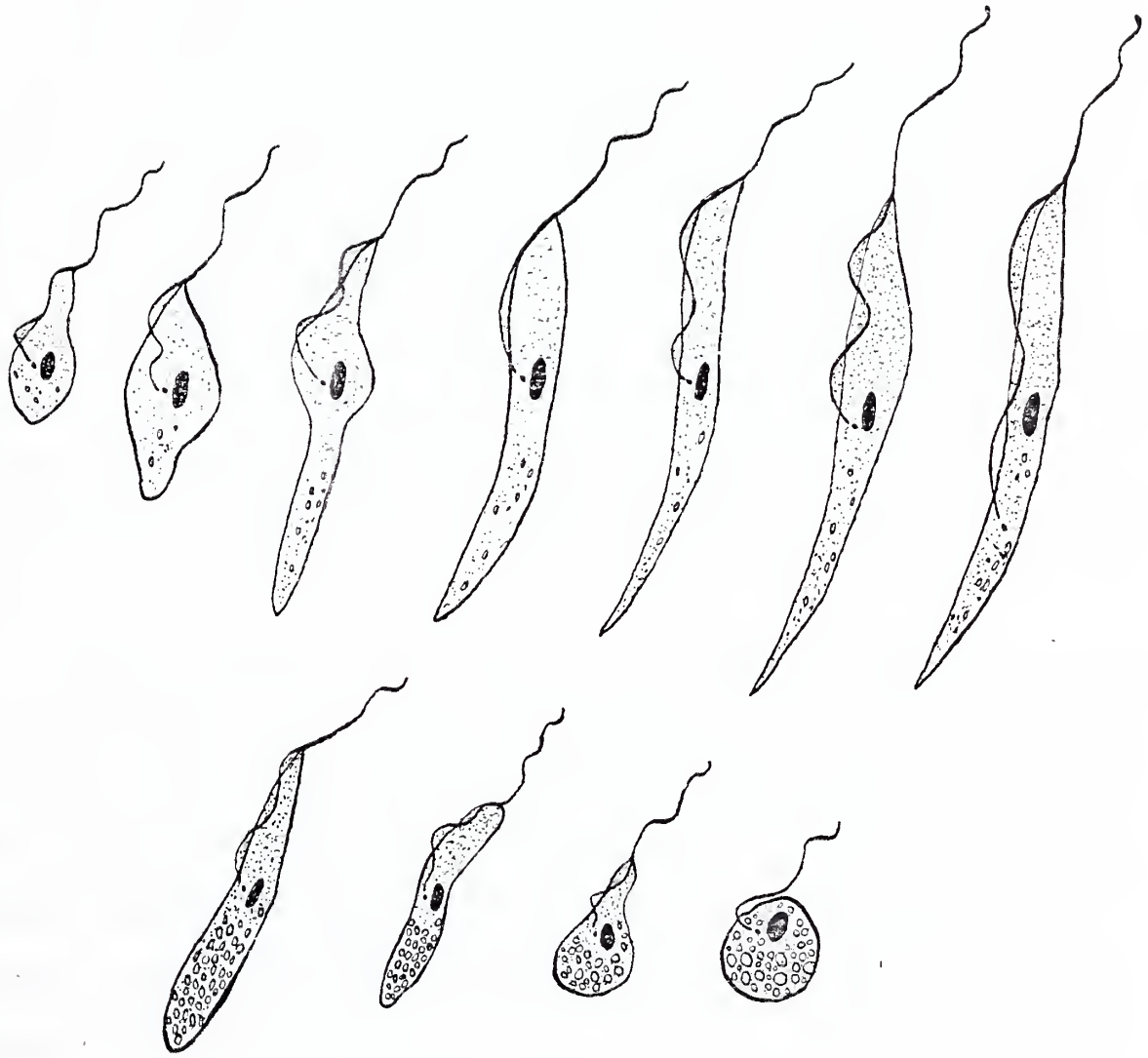


Figure 23. Epimastigote and Trypomastigote Forms Observed in Flagellate Cultures. Top row: Developing epimastigotes and trypomastigote. Bottom row: Degenerate vacuolated epimastigotes. Drawn to scale: 2 mm. ≅ 1 μ .

after transfer to new medium, elongate typical epimastigote forms were observed (Figure 24). After about 3 weeks, aberrant, rounded, degenerate forms appeared (Figure 25).

The typical elongate epimastigote form observed (Figures 26-28) had the following characteristics: a pointed posterior end, a nucleus about 1/3 the length of the cell distant from the anterior end, a small knob on the tip of the free flagellum and cytoplasmic inclusions of two types, refractile vacuoles and solid particles. The degree to which the posterior end was pointed and the prominence of the flagellar knob were variable. Early epimastigote cultures also exhibited a "club" form characterized by a bulge at the level of the nucleus (Figures 23, 29).

Giemsa-stained organelles of epimastigotes were very clearly delineated: the nucleus was stained deep purple; the kinetoplast, dark blue to black; the flagellum, dark blue; and the cytoplasm, pale blue. Particulate inclusions in the cytoplasm stained dark blue and vacuolar inclusions appeared as clear circular areas highlighted by the pale blue cytoplasm around them.

As a culture aged, the epimastigotes exhibited larger numbers of vacuoles (Figures 23, 30, 31). The posterior ends of older epimastigotes became less pointed as vacuoles filled the cytoplasm and distended the sides of the cells. The epimastigotes became shorter and more rounded (Figures 23, 32). These small, degenerate epimastigotes eventually were completely distorted by the presence of large vacuoles which compressed the

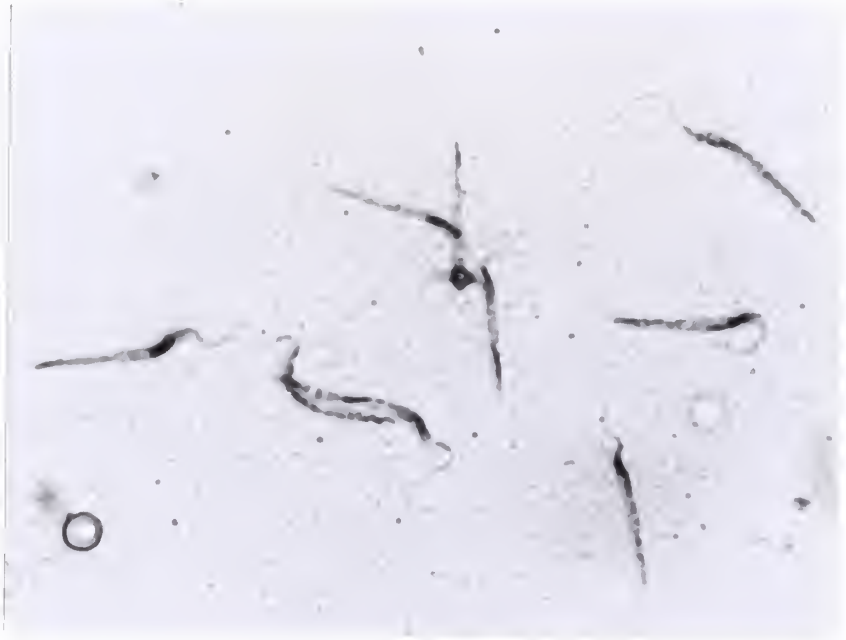


Figure 24. Epimastigotes (tabanid clone culture, N.N.N. 28°). 900 X.

Figure 25. Epimastigotes - diverse forms (tabanid clone culture, N.N.N. 28°). 900 X.

Figure 26. Epimastigotes (tabanid clone culture, N.N.N. 28°). 3000 X.

Figure 27. Epimastigotes and rabbit erythrocyte (tabanid clone culture, N.N.N. 28°). 2000 X.

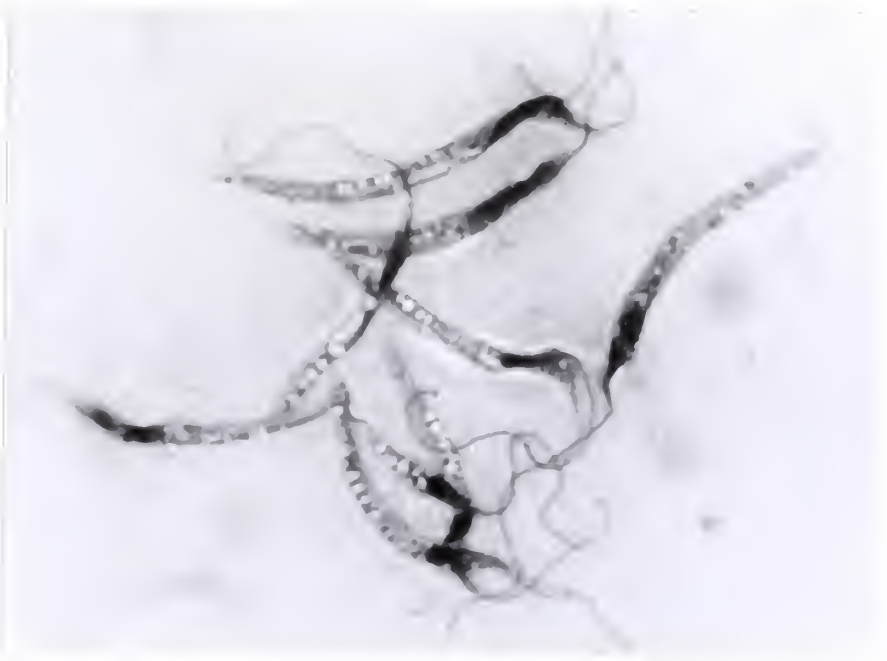


Figure 28. Epimastigotes (tabanid clone culture, N.N.N. 28°). 2000 X.

Figure 29. Epimastigote - "club" form (tabanid clone culture, N.N.N. 28°). 3000 X.

Figure 30. Epimastigote (tabanid clone culture, N.N.N. 28°). 3000 X.

Figure 31. Epimastigotes - degenerating (tabanid clone culture, N.N.N. 28°). 3000 X.

Figure 32. Epimastigote - degenerate (tabanid clone culture, N.N.N. 28°). 3000 X.

Figure 33. Epimastigote - degenerate (end stage) (tabanid clone culture, N.N.N. 28°). 3000 X.

contents of the cells (Figures 23, 33).

Degenerating forms did not stain as well with Giemsa as the early typical forms. The organelles were pale and the flagellum often did not stain at all. The color of the nucleus ranged from purple to pale pink, depending on the degree of nuclear degeneration (karyorrhexis).

The transformation of elongate into shorter flagellate forms led to a wide variation in the size of the flagellates. Quantitative observations of cultured flagellates are presented below.

(2) Quantitative Observations. N.N.N. cultures of flagellates derived from tabanids collected in 1972 (Table XXVII) were used to estimate the size of epimastigote forms. A constant aliquot of each culture was transferred to fresh medium every 14 days and measurements were made every 7 days. In Figures 34-36, epimastigotes in each size range are presented as a percentage of the cultures in which they were observed. Epimastigotes ranged in size from 10μ to 70μ in total length (L). The majority of organisms were approximately 20-30μ in length and 2-4μ in width. Flagellates in cultures from Chrysops, Hybomitra, and Tabanus species fell within this size range (Figure 37).

Giemsa-stained preparations of fixed epimastigotes were used for the determination of exact measurements. The body lengths (PN + NA) of 25 living epimastigotes in samples from each of 4 N.N.N. cultures were measured. Each sample was then fixed and stained. A sample of 25 fixed flagellates from each

Figure 34. Size of Epimastigotes Cultured from Chrysops Species (1972)

(% of cultures in which each size range was observed)
N = number of cultures examined

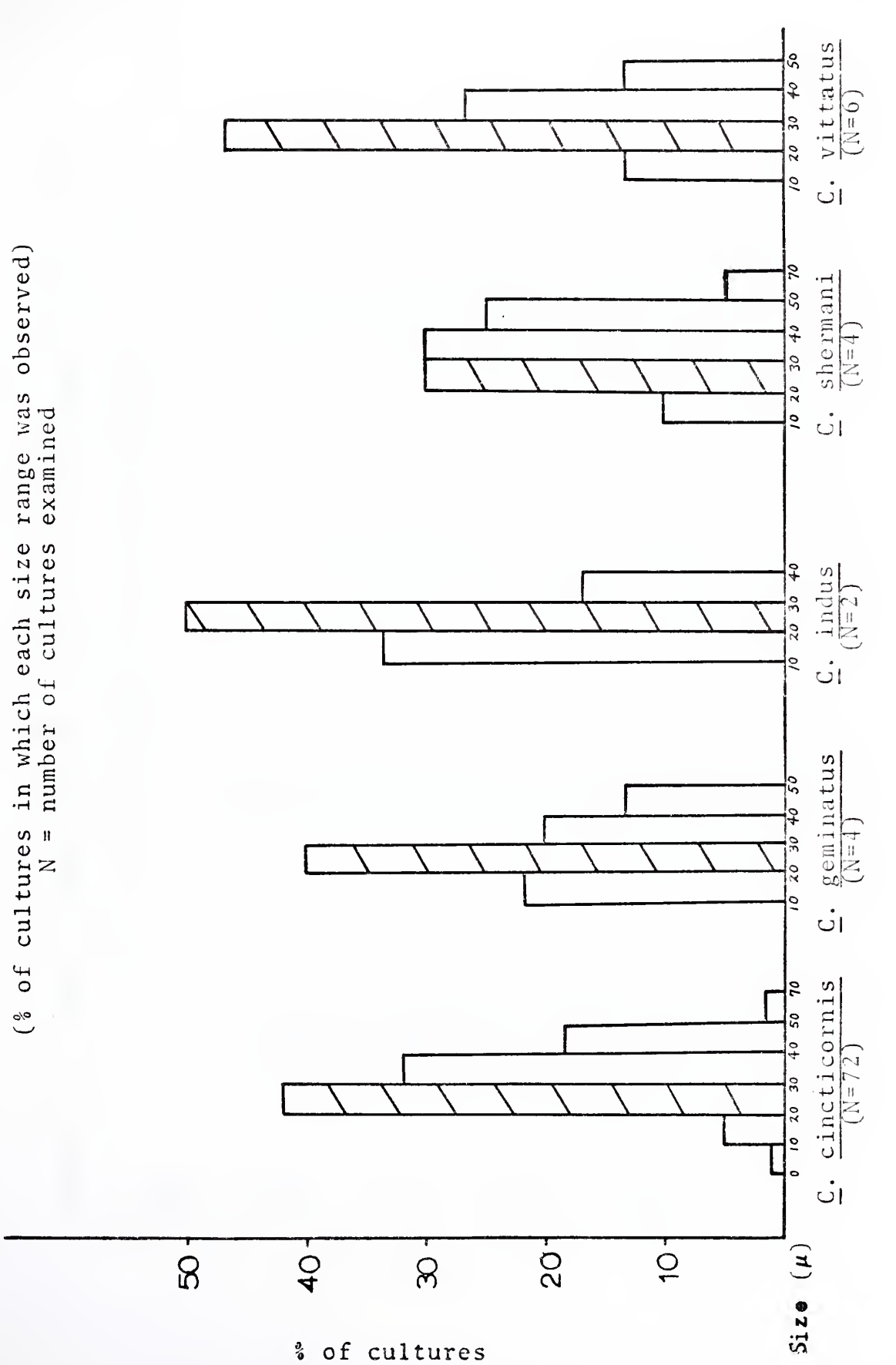


Figure 35. Size of Epimastigotes Cultured from Hybomitra Species (1972)

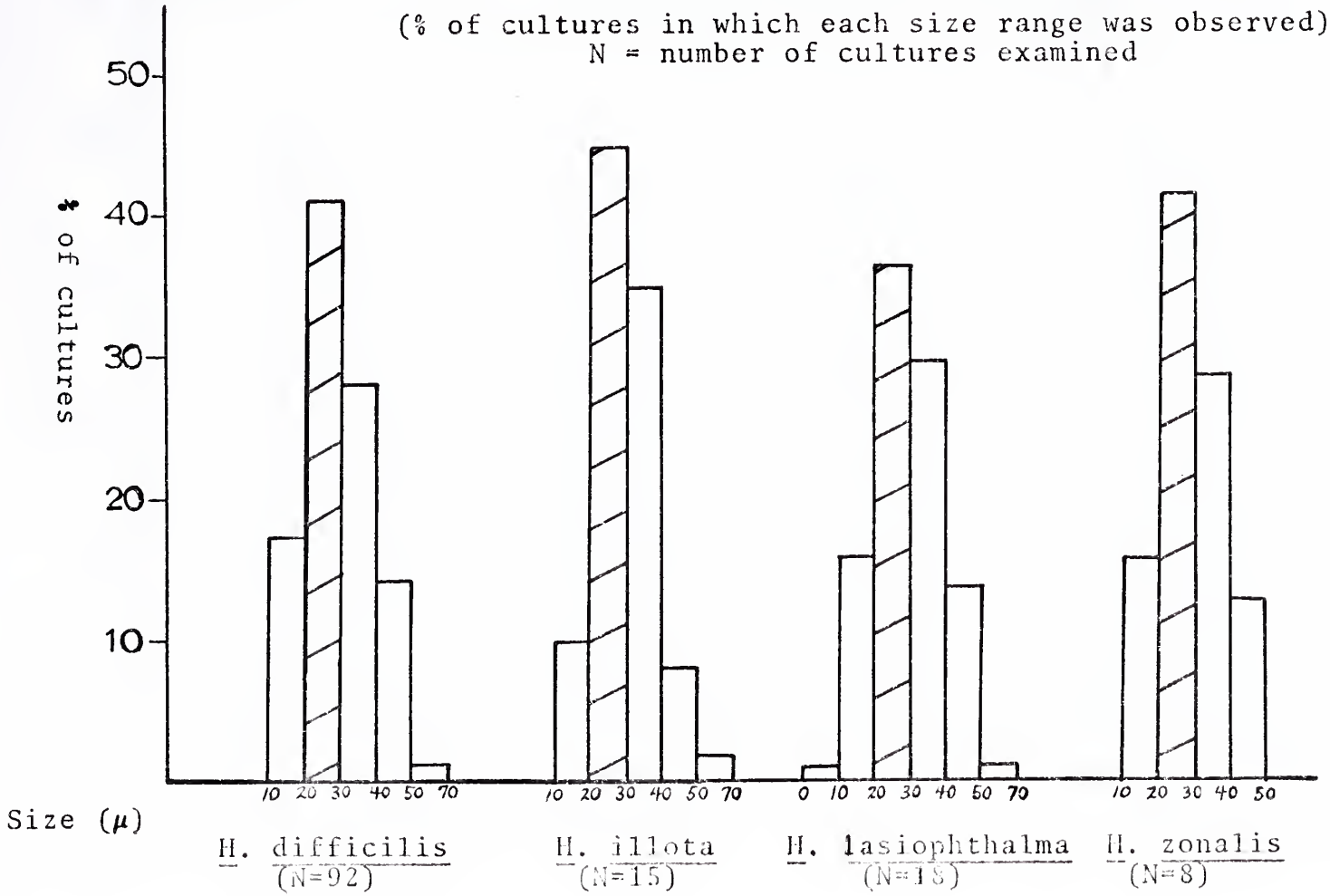
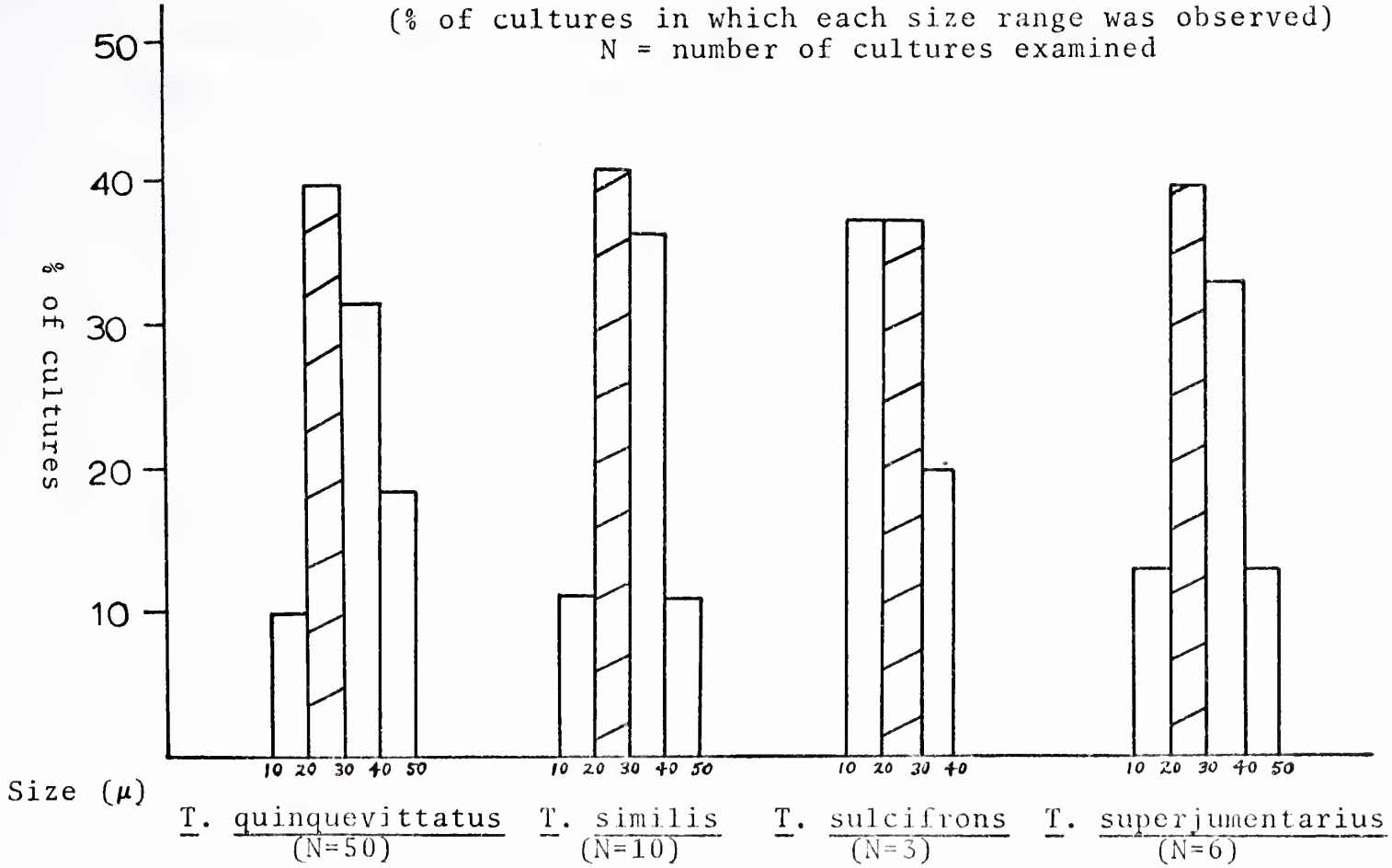


Figure 36. Size of Epimastigotes Cultured from Tabanus Species (1972)



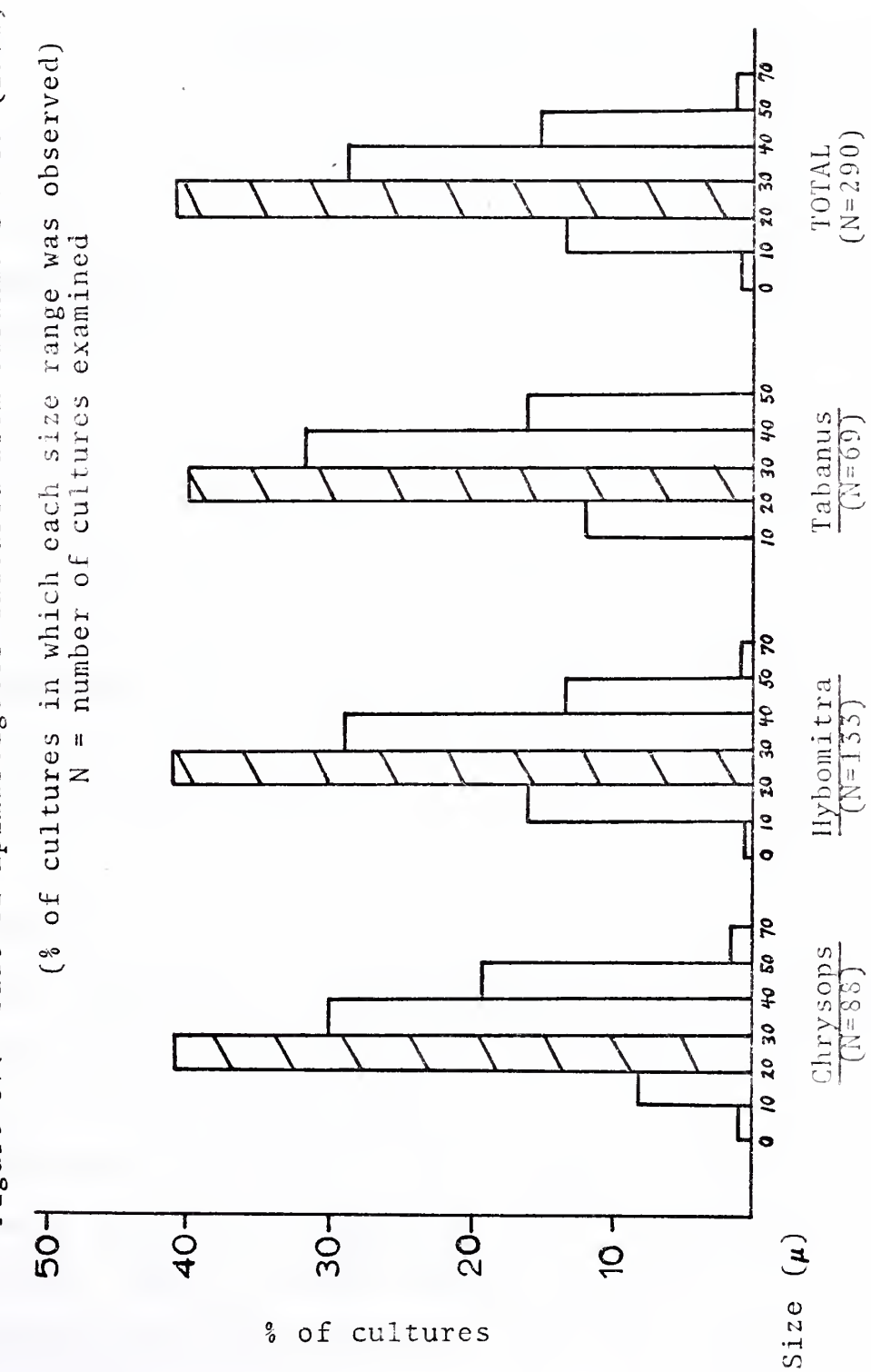


Figure 37. Size of Epimastigotes Cultured from Tabanid Genera (1972)
(% of cultures in which each size range was observed)
N = number of cultures examined

of these four culture samples was then measured. The results are listed below:

Culture	Average Body Length (Range of Body Lengths) Unfixed	Fixed
C. cincticornis	14 (5-18)	13 (10-17)
H. difficilis	12 (10-21)	12 (7-17)
T. superjumentarius	15 (10-23)	16 (10-25)
T. quinquevittatus	13 (7-25)	14 (8-21)

The comparison of measurements indicated that fixing did not affect the size of flagellates.

Fixed flagellates grown in different media (N.N.N. and McCoy's) and from cultures of different ages were compared. The mean values and ranges of these measurements are presented in Table XXXII. The size of the epimastigotes observed was dependent on the culture medium used and the age of the culture sampled.

A comparison of N.N.N. and McCoy's cultures of the same age (see H. difficilis, T. superjumentarius and T. quinquevittatus, Table XXXII), indicated that epimastigote forms tend to be larger in McCoy's medium than in N.N.N. This was graphically demonstrated by plotting the size ranges of flagellates from two of these samples, T. difficilis and T. superjumentarius (Figure 38 A). McCoy's medium favored the growth of epimastigotes over 30μ in total length (L), while the predominant forms in N.N.N. cultures from the same species were less than

Table XXXII. Tabanid Flagellates - Fixed, Giemsa-Stained Epimastigote Culture Forms

Culture[2]	Age[3] (days)	N	$\bar{X}$[1] PK	PN	KN	NA	F	KI	NI	L
N.N.N. 28°										
H. difficilis	6	25	10 (5-14)	8 (5-13)	<1 (0≤1)	6 (3-9)	12 (8-18)	>5	1.5 (0.8-2.3)	27 (18-33)
T. superjumentarius	4	25	9 (3-15)	9 (4-15)	<1 (0≤1)	7 (5-12)	12 (6-19)	>5	1.3 (0.6-2.5)	28 (20-37)
T. quinquevittatus (clone)	3	50	14 (3-22)	12 (2-24)	<1 (0≤1)	6 (3-11)	16 (7-25)	>5	2.3 (0.2-4.0)	34 (22-48)

[1]Mean ($\bar{X}$) measurements are given in microns (μ) for a number (N) of flagellates in each culture. Definitions for parts measured (PK, PN, etc.) are given in the text and Figure 9. The range of values for each measurement is given in μ in parenthesis under the $\bar{X}$ value.

[2]Cultures with the same species name were derived from the same fly specimen.

[3]Age refers to days since transfer, not since initiation of the original culture.

Table XXXII, Continued

Culture	Age (days)	N	$\bar{X}$ PK	PN	KN	NA	F	KI	NI	L
McCoy's 28°										
C.cincticornis	6	25	11 (6-20)	10 (5-19)	<1 (0≤2)	6 (4-8)	15 (6-24)	>5	1.6 (1.0-2.7)	32 (16-43)
H.difficilis	6	25	11 (7-15)	10 (7-14)	<1 (0≤1)	6 (4-9)	15 (10-20)	>5	1.6 (1.0-3.0)	30 (24-40)
T.superjumentarius	4	25	10 (3-15)	9 (5-14)	<1 (0≤1)	10 (5-15)	13 (9-23)	>5	1.0 (0.3-1.4)	32 (25-46)
T.quinquevittatus (clone)	2	25	18 (12-23)	16 (11-21)	<1 (0≤1)	8 (5-14)	14 (8-26)	>5	1.9 (1.2-4.0)	40 (26-51)
T.quinquevittatus (clone)	14	25	12 (3-16)	10 (2-21)	<1 (0≤1)	6 (3-10)	16 (10-28)	>5	1.6 (0.6-3.2)	33 (23-48)

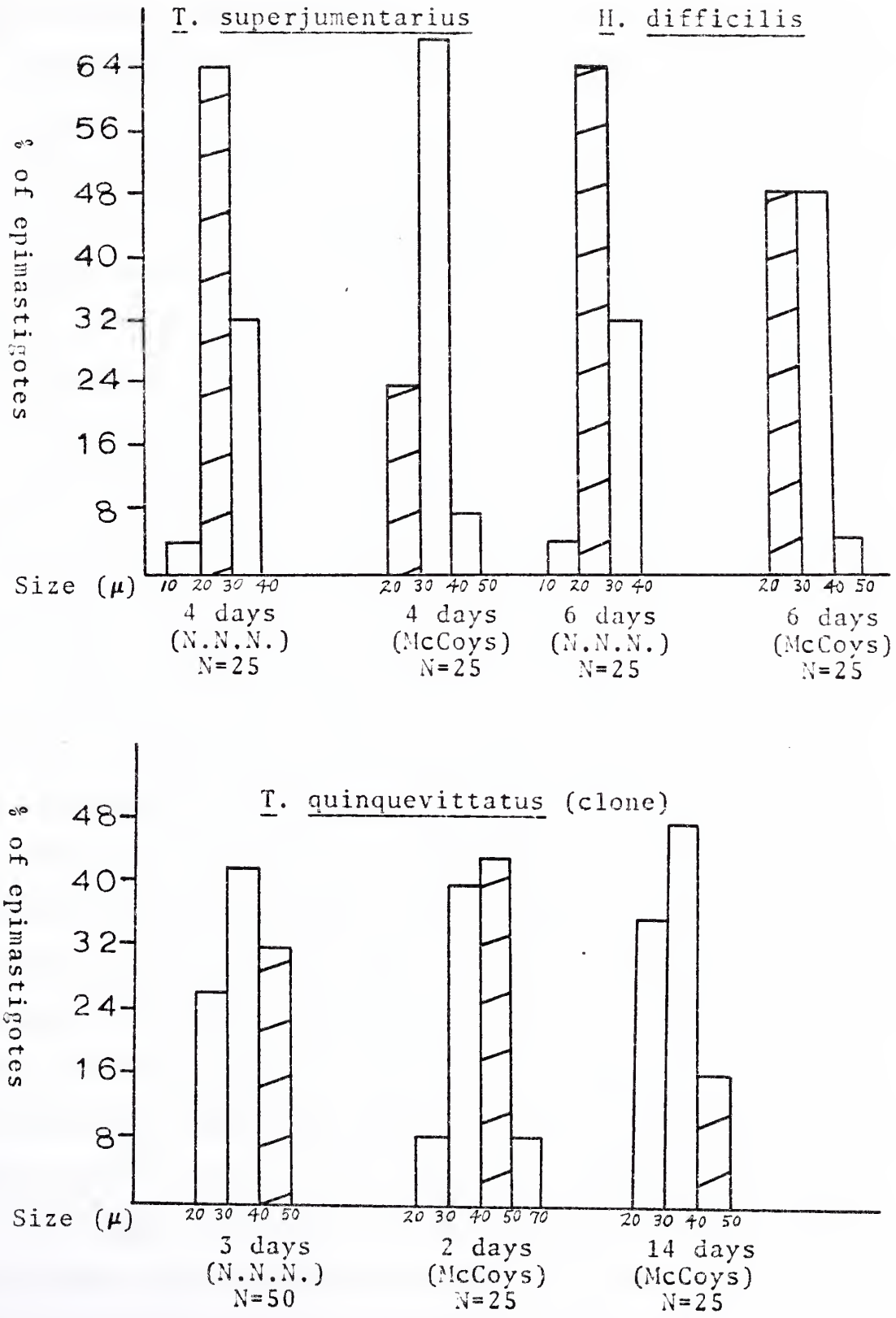


Figure 38. Effects of Medium Type and Culture Age on Size of Flagellates Grown at 28°

30 μ in length. Fixed specimens from all species listed in Table XXXII ranged in total length (L) from 16-51 μ and in width from 1.5-5 μ .

Recently transferred cultures exhibited larger forms than older cultures. Thus, T. quinquevittatus (clone) cultures at 2 and 3 days post-transfer (Table XXXII) had larger forms than cultures in the same media at 4-14 and 4-6 days post-transfer. This difference is graphically illustrated in Figure 38 B.

A difference in total length (L) of epimastigotes from young and old cultures was accompanied by a difference in the position of the nucleus. Both young cultures of T. quinquevittatus (2 and 3 days post-transfer) exhibited epimastigotes with nuclei closer to their anterior ends ($\overline{X}_{NI}$ = 1.9 and 2.3) than those in older cultures ($\overline{X}_{NI}$ = 1.0-1.6) (Table XXXII). This difference is consistent with the morphological observation that as an epimastigote degenerates, vacuolar inclusions are most abundant in the posterior portion of the cell, resulting in a widening and shortening of the posterior end. As the posterior end of the cell shortens, the nucleus, though remaining at a constant distance from the anterior end, appears to be moving closer and closer to the middle of the cell. The $\overline{X}_{NA}$ values for all cultures in Table XXXII are very similar while the $\overline{X}_{PN}$ values of the 2- and 3-day old T. quinquevittatus cultures are larger than those of older cultures. This comparison indicates that the apparent change in position of the nucleus is a function of the shortening of the length of the cell from the posterior end rather than a shift in the position of

the nucleus itself.

In general, the measurements of all stained epimastigotes (Table XXXII) were consistent with the morphological observations of typical epimastigote forms mentioned earlier, i.e., they have a nucleus either at the middle or anterior end of the cell (NI $\geq$ 1.0) and a kinetoplast closer to the nucleus than to either end of the cell (KI > 5).

The diverse morphological forms of epimastigotes observed in tabanid cultures were identical to epimastigotes of T. theileri-like flagellates cultured from cattle and described and illustrated by Crawley (1909, 1912), Glaser (1922) and Herbert (1965b). The size range of the tabanid culture forms was consistent with that of dividing (epimastigote) forms (18-70 μ) of T. theileri-like flagellates reported by others (Laveran and Mesnil, 1907; Curasson, 1943; Herbert, 1965b).

Trypomastigote Forms. As described earlier, three cultures exhibited small numbers of trypomastigotes (CuF_{10} 37° and McCoy's (clone) 28°). Because of their unique movements, these forms were readily apparent in living cultures. Whereas all tabanid flagellates seen previously in cultures progressed with their anterior ends forward in a rapid fashion, the trypomastigotes exhibited a less progressive side-to-side movement. This swaying movement, characteristic of adult Trypanosoma species, was apparently related to the length of the undulating membrane. The membrane of the trypomastigotes was slightly more prominent than the narrow, short membrane exhibited by epimastigotes in all other cultures. Some of the epimastigotes in

the cultures harboring trypomastigotes exhibited a combination of the forward progression and side-to-side movement. Closer examination of these forms revealed elongate epimastigotes, each with a kinetoplast at the level of the posterior end of the nucleus. These were probably transitional forms between epimastigotes and trypomastigotes.

The fixed specimens of trypomastigotes exhibited the same staining properties as epimastigotes. The major difference in morphology between epimastigotes and trypomastigotes was the characteristic posterior displacement of the kinetoplast. The trypomastigotes observed had the nucleus closer to the middle of the cell and the kinetoplast closer to the nucleus than to the posterior end. The trypomastigote forms were 35-45 μ long (L) with pointed posterior ends and exhibited fine vacuolar and granular cytoplasmic inclusions (Figure 39).

The relatively large size of the trypomastigotes and the position of the kinetoplast closer to the nucleus than to the posterior end are diagnostic characteristics of the Trypanosoma subgenus Megatrypanum Hoare 1964. In size, shape and internal morphology, the trypomastigotes observed were similar to the blood forms of T. theileri-like trypanosomes isolated from ruminants.

Choanomastigote Forms. The choanomastigote forms observed in culture were similar to Type B forms (Figure 10) seen in the rectum of one specimen of T. marginalis (Section II A). The two pure cultures (McCoy's medium 28^{o}) obtained in 1973 were studied in detail. Measurements of samples from each of these

Figure 39. Trypomastigote (tabanid clone culture, McCoy's 28°). 3000 X.

cultures are presented at the beginning of each of the following sections in which the flagellates are described. The measurements are given in μ and the abbreviations (except for KI) are defined in Figure 9. Since each choanomastigote had a kinetoplast anterior to the nucleus, the KI = NA/KN. Using the latter definition, a value of KI > 2 indicates a kinetoplast closer to the nucleus than to the anterior end of the cell.

(1) Crithidia sp. # 1 (from T. marginalis).

N	PK	PN	KN	NA	F	KI	NI	L
50	4 (2-8)	4 (1-7)	<1 (0≤1)	4 (2-6)	6 (2-11)	>3	1.0 (0.2-2.3)	13 (8-19)

These choanomastigotes are characterized by a form with a rounded or truncated anterior end having little or no constriction behind the opening of the reservoir. The posterior end is very polymorphic being either rounded, swollen or drawn out to a point (Figures 40, 41). The nucleus is in the middle of the cell and the kinetoplast occupies a laterial position 1/3 to 1/2 the body length from the anterior end. The flagellum runs through a conspicuous tubular reservoir (Figure 42).

These forms grew in a culture inoculated with a small number of choanomastigotes which were found swimming free in the lumen of the rectum of a tabanid, T. marginalis (Section II A). The fly was a non-blooded, parous female captured at Site VI with an insect net.

Figure 40. Crithidia sp. # 1 (McCoy's culture 28°, Source: T. marginalis collected at Site VI, 1973). 3000 X.

Figure 41. Crithidia sp. # 1 (Same data as Figure 40). 3000 X.

Figure 42. Crithidia sp. # 1. Note tubular reservoir with flagellum running through it. (Same data as Figure 40.). 3000 X.

Figure 43. Crithidia sp. # 2 (McCoy's culture 28°, Source: blooded T. quinquevittatus collected at Site V, 1973). 3000 X.

(2) Crithidia sp. # 2 (from T. quinquevittatus).

N	PK	PN	KN	NA	F	KI	NI	L
50	4 (3-6)	2 (1-4)	<1 (0≤1)	5 (3-7)	4 (2-8)	>2	0.6 (0.2-2.0)	11 (8-16)

These choanomastigotes are characterized by an ovoid to bullet-shaped form with a rounded or blunt anterior end having no constriction behind the opening of the reservoir. The posterior end is tapered to a rounded or slightly pointed tip (Figures 43, 44). The nucleus is 1/6 to 1/2 the body length from the posterior end (Figure 45). The kinetoplast occupies a lateral position 1/3 to 1/2 the body length from the anterior end. The flagellum runs through a short funnel-shaped reservoir.

These choanomastigotes grew in a culture inoculated with Type I and II amastigotes (Section II A) from the hindgut of a specimen of T. quinquevittatus. The initial culture exhibited choanomastigote and epimastigote forms but, after 21 days, only choanomastigote forms were observed. The fly was a nulliparous, blooded female captured in the canopy trap at Site V.

The Crithidia sp. #2 differs from the preceding one in the following ways: its total length (L) is shorter, the nucleus tends to be closer to the posterior end, and the shape is less polymorphic than Crithidia sp. # 1.

Amastigote Forms. Amastigotes were seen in 5 replicates of 2 cultures which had been maintained by subculturing for several months (H. illota - 11 months old; T. quinquevittatus - 3, 4, 4½, 5½ months old). Small non-flagellated forms which

Figure 44. Crithidia sp. # 2 (Same data as Figure 43). 3000 X.

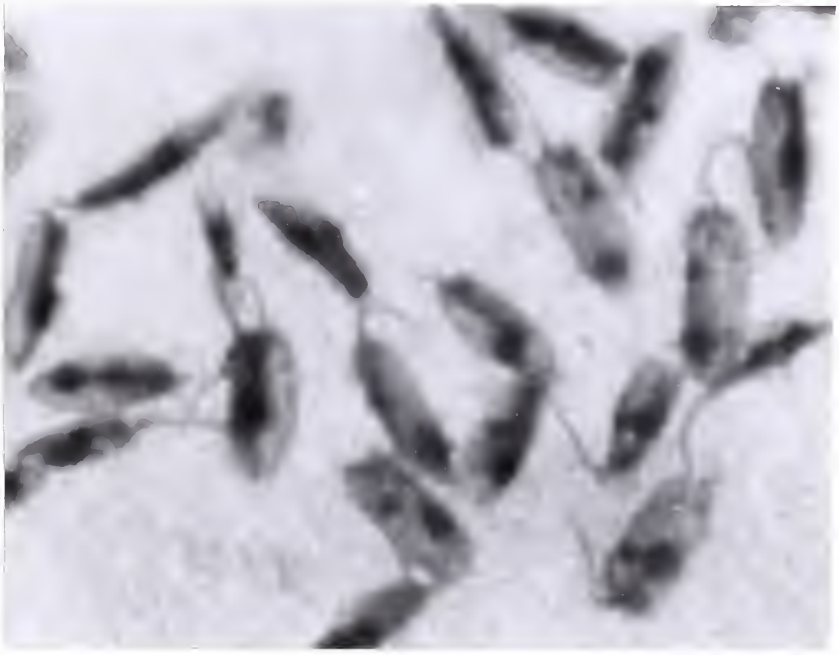


Figure 45. Crithidia sp. # 2 (Same data as Figure 43). 3000 X.

resembled the Type I amastigotes observed in tabanid alimentary tracts were seen. The forms measured 4-8 μ in length and 2-4 μ in width and possessed a juxtanuclear kinetoplast. Each flagellate had a tapered anterior end and granular cytoplasm (Figure 46). The exact nature of these forms is uncertain. Their small size and "barley corn" shape are consistent with the forms seen in tabanids rather than the degenerate epimastigotes seen in other older cultures (Figures 23, 25). The T. quinquevittatus used to prepare 4 of these cultures was the same as that used to prepare the epimastigote culture in which trypomastigote forms appeared.

B. Growth of Flagellate Forms in Culture

Motile forms were never observed in cultures inoculated with immotile forms from tabanids earlier than 48 hours after inoculation. Small epimastigote forms and forms resembling flagellated immotile forms (8-15 μ = L) were observed at 48-72 hours after inoculation. Large numbers of typical epimastigote forms (15-35 μ = L) were commonly observed 4-10 days after initiation of cultures with infected tabanid tissues.

The numbers of flagellates in 3 replicates of a clone culture (from T. quinquevittatus) were measured every 2-3 days for 26 days using a hemocytometer (Figure 47). The cultures were not transferred, and no fresh media was added during the counting study. Each study was terminated when all of the culture medium in each tube had been utilized for counting.

Figure 46. Amastigote in culture (N.N.N. 28°, Source: T. quinquevittatus collected at Site IV A, 1972). 3000 X.

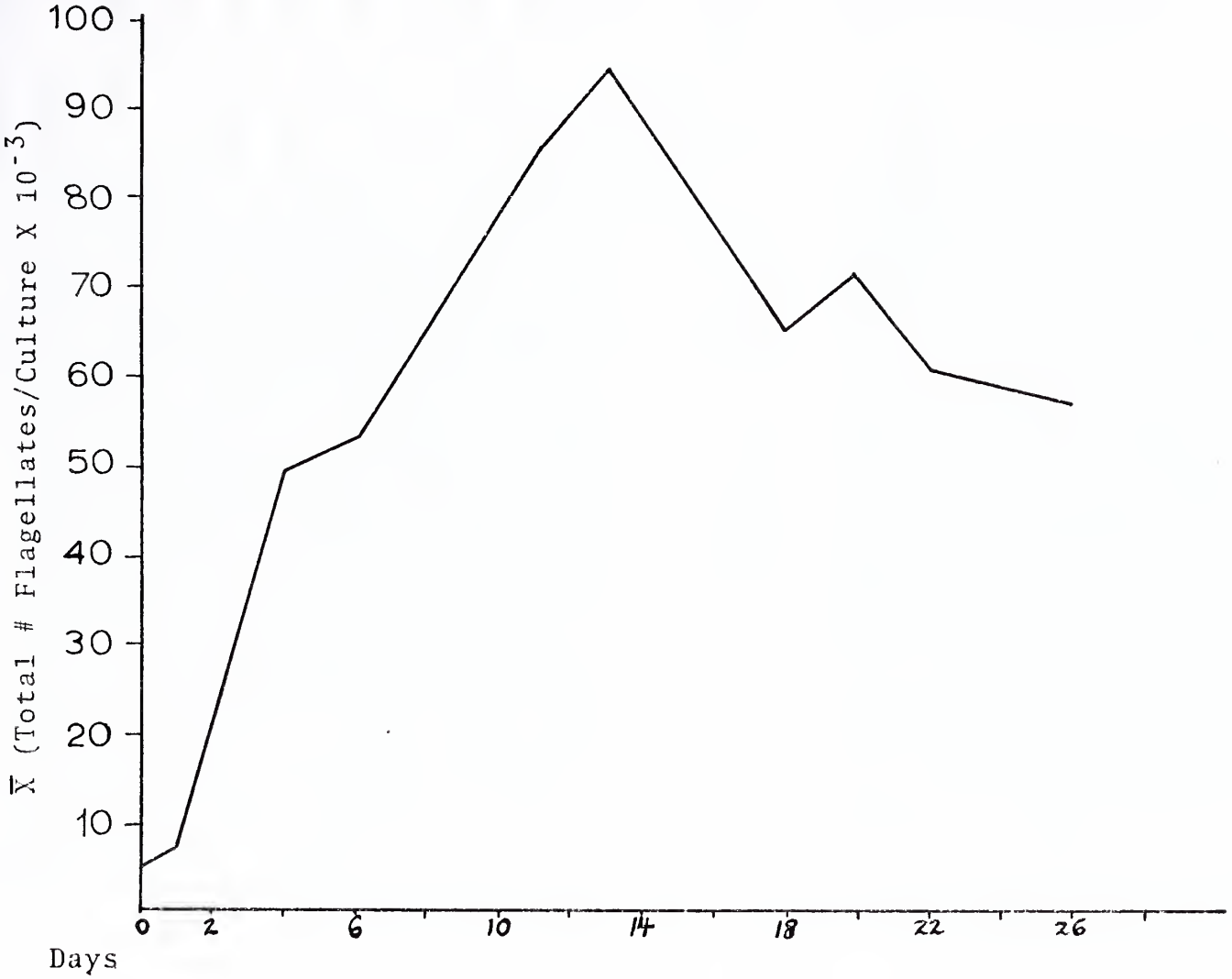


Figure 47. Growth of Clone Flagellates (T. quinquevittatus) in N.N.N. Medium (28°) (N=3 cultures)

Figure 47 indicates that the flagellate forms exhibited the same general growth curve as that seen in cultures of other microorganisms, i.e., a slight lag phase and a period of rapid growth followed by a population decline.

In general, tabanid flagellates were able to survive in culture for long periods without the addition of fresh media. Twenty-one N.N.N. cultures (28^{o}) representing 9 species of tabanids remained viable with epimastigote forms for 8 weeks without the addition of fresh media. Similarly, clone cultures (T. quinquevittatus) in McCoy's medium (28^{o}) were observed to survive for 4-6 weeks without being supplemented.

C. Division of Flagellate Forms in Culture

Only epimastigote forms were observed to reproduce in culture. Binary fission commenced with division of the nucleus and kinetoplast. The resulting duplicate organelles of each type were oriented in a row along the anterior-posterior axis of the cell. Following division of the organelles and growth of a new flagellum, the cytoplasm divided (Figure 48), producing two daughter cells. Uneven division resulting in two cells of different sizes (Figure 49) was common. Binary longitudinal fission of epimastigotes of diverse shapes was noted, as in the dividing rounded forms in Figure 50.

Rapidly growing cultures exhibited epimastigotes in rosette formations (Figure 51). The flagellates were arranged with their anterior ends toward the center of each rosette.

Figure 48. Dividing epimastigote. Note knobbed flagella. (tabanid clone culture, N.N.N. 28°). 3000 X.

Figure 49. Unequal division of epimastigote (McCoy's culture 28°, Source: Chrysops cincticornis collected at Site I, 1972). 3000 X.

Figure 50. Unequal division of epimastigote (McCoy's culture 28°, Source: Hybomitra difficilis collected at Site II, 1972). 3000 X.

Figure 51. Rosette in culture (tabanid clone culture, McCoy's 28°). 900 X.

Rosettes of different sizes and density were observed, as in Figures 52 and 53. Coalescence of rosettes occasionally produced monolayers of cells on the surface of the agar of the N.N.N. medium.

The growth and division of epimastigotes in culture was consistent in all respects with descriptions of the cultural characteristics of epimastigote forms of T. theileri-like epimastigote forms (Hoare, 1972).

IV. Frozen Storage of Trypanosomes

Trypanosomes grown in McCoy's medium were successfully recovered as viable organisms after freezing in 10% glycerol at -70°. Cultures grown in McCoy's medium at 28° (clone from T. quinquevittatus, T. lewisi, Crithidia spp. # 1 and 2) and at 37° (T. theileri type culture) exhibited lively organisms when thawed ten weeks after freezing. Splitter and Soulsby (1967) obtained similar results with cultures of T. theileri.

V. Flagellate Cultures from Ruminants

A. T. theileri-like Trypanosomes from a Cow

A trypanosome culture (CuF_{10} - 37°) from a dairy cow exhibited epimastigotes and trypomastigotes typical of the subgenus Megatrypanum. The flagellates were 20-40 μ in total length (L) and progressed in a side-to-side fashion. Each trypanosome had a pointed posterior end and a short free flagellum.

Figure 52. Rosette in culture (McCoy's 28°, Source: Hybomitra difficilis collected at Site II, 1972). 1350 X.

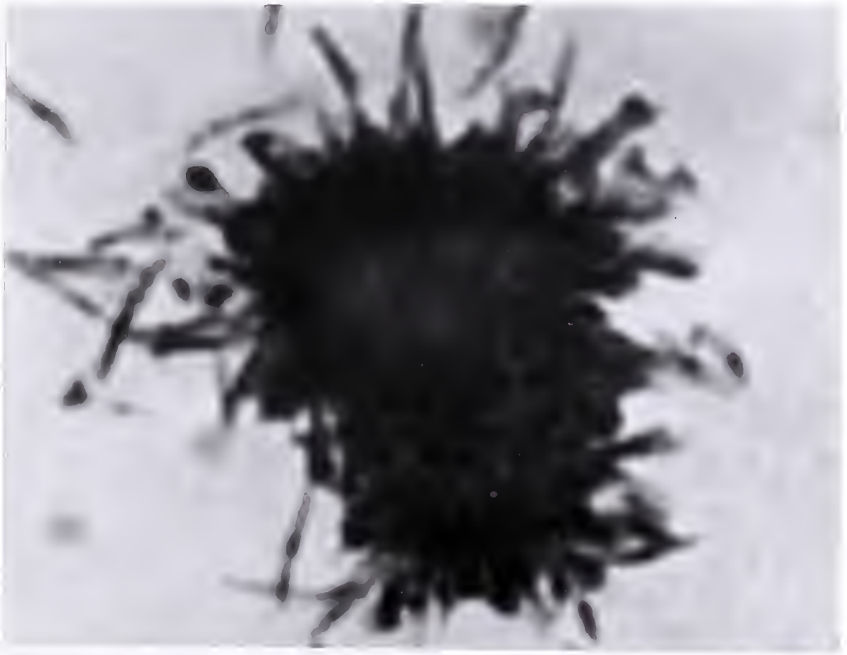


Figure 53. Rosette in culture (Same data as Figure 52). 1350 X.

The nucleus was 1/2 to 1/6 the body length from the anterior end of the cell. The kinetoplast was closer to the nucleus than the posterior end of the cell. The cytoplasm was granular. After 10 days, the culture exhibited mostly epimastigote forms, many of which were small and rounded. These degenerate forms were similar to the flagellates seen in older tabanid cultures (Figures 23, 25).

In size, shape and internal morphology, the flagellates isolated from the dairy cow were indistinguishable from T. theileri Laveran (1902a) and T. theileri-like trypanosomes from dairy cattle (Woo et al., 1970; Wells, 1971b; Hoyte, 1972).

B. T. theileri Laveran 1902 Type Culture

The type culture obtained from the American Type Culture Collection exhibited short, granular epimastigotes and trypomastigotes with short free flagella (Figures 54 - 56). These forms were maintained in McCoy's medium (37°) with transfers every 4-5 days. Measurements of 100 fixed, giemsa-stained flagellates revealed a mean body length of 10 μ (range, 5-16 μ) and a free flagellum of 5 μ (range, 2-9 μ). The total length (L) ranged from 9-21 μ.

Binary fission of T. theileri began with division of the nucleus and kinetoplast (Figures 57 and 58) followed by division of the cell (Figures 59 and 60). The presence of forms with two nuclei, one anterior to the other, and one flagellum was consistent with observations of recently isolated T.

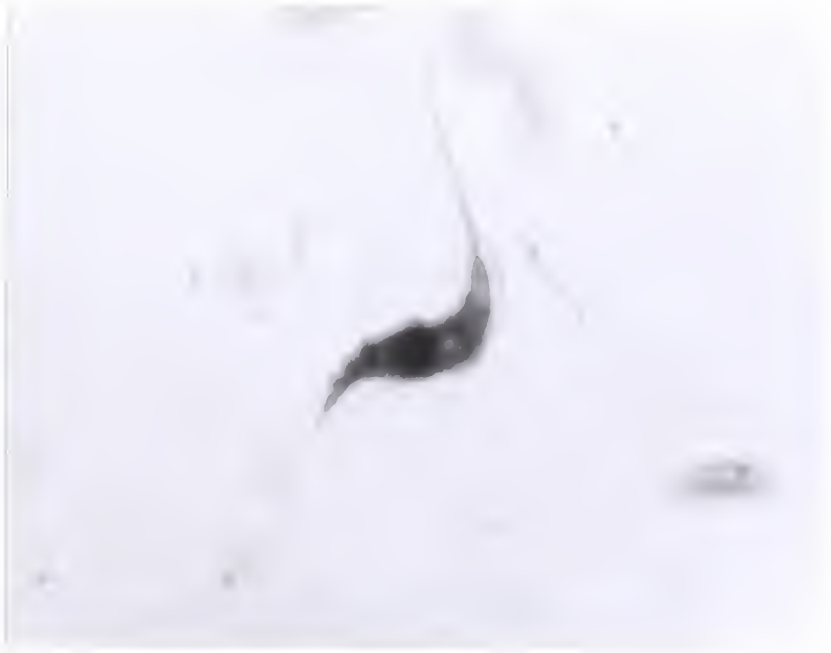


Figure 54. Trypanosoma theileri (trypomastigote) (Type Culture McCoy's 37°). 3000 X.

Figure 55. Trypanosoma theileri (trypomastigote) (Type Culture McCoy's 37°). 3000 X.

Figure 56. Trypanosoma theileri (epimastigote) (Type Culture McCoy's 37°). 3000 X.

Figure 57. Trypanosoma theileri (early division stage of epimastigote)(Type Culture McCoy's 37°). 3000 X.

Figure 58. Trypanosoma theileri (dividing epimastigote) (Type Culture McCoy's 37°). 3000 X.

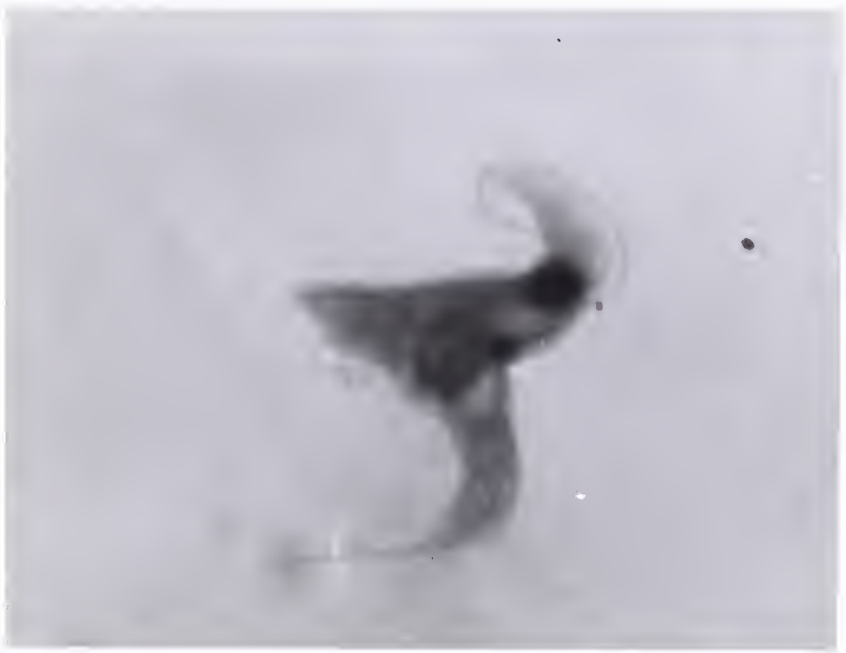


Figure 59. Trypanosoma theileri (dividing epimastigote) (Type Culture McCoy's 37°). 3000 X.

Figure 60. Trypanosoma theileri (last stage of division of epimastigote)(Type Culture McCoy's 37°). 3000 X.

theileri-like organisms (Wells, 1971a). Epimastigotes were the only forms observed dividing by binary fission.

In size, shape and internal morphology, the type culture forms resembled the smaller forms of T. theileri described by Theiler (1903).

C. T. theileri-like Trypanosomes from White-tailed Deer

Five white-tailed deer (Odocoileus virginianus) from the New York State Game Farm (Ithaca) were bled from the jugular vein at irregular intervals. The schedule of bleedings and the blood culture results are presented in Table XXXIII. These observations of flagellates from deer represent the first isolations of trypanosomes from white-tailed deer in the Northeastern United States. Wet and fixed blood smears of each sample failed to demonstrate flagellates. Uninoculated culture tubes from the same preparation batches as those inoculated failed to exhibit any flagellate growth.

Flagellate cultures at 37° exhibited more diverse, aberrant epimastigote forms than those grown at 28° (cf. Figure 61 with Figures 62 and 63). Measurements of fixed, giemsa-stained epimastigotes are presented in Table XXXIV. As in the case of tabanid flagellates (Table XXXII), flagellates grown in N.N.N. at 28° (or McCoy's at 37°) were shorter than those grown in McCoy's at 28°. Similarly, the 3-day old cultures exhibited larger forms than the 10-day old cultures. No trypomastigote forms were observed. In general, the epimastigotes from deer

Table XXXIII. Trypanosome Isolations from White-tailed Deer

Date (1973)	Deer (#)	Age (yrs)	Sex	Culture Media	
				N.N.N. (28°)	McCoy's (37°)
26 March	3	1½	M	Negative	Negative
26 March	18	1½	F	Negative	Negative
17 July	16	2	F	Epimastigotes(at 4 days)	Epimastigotes(at 14 days)
7 August	3	2	M	Epimastigotes(at 3 days)	Negative
30 August	3	2	M	Epimastigotes(at 6 days)	Epimastigotes(at 13 days)
10 September	3	2	M	Epimastigotes(at 10 days)	Epimastigotes(at 10 days)*
10 September	60	4¼	M	Epimastigotes(at 10 days)	Epimastigotes(at 10 days)*
10 September	81	1¼	M	Epimastigotes(at 18 days)	Epimastigotes(at 10 days)*

*These forms were isolated at the same time in McCoy's (28°).

Figure 61. T. theileri-like trypanosomes from Deer # 3 (McCoy's culture 37°). 3000 X.

Figure 62. T. theileri-like trypanosome (epimastigote) from Deer # 81 (McCoy's culture 28°). 3000 X.

Figure 63. T. theileri-like trypanosome (early stage of dividing epimastigote) from Deer # 81 (McCoy's Culture 28°). 3000 X.

Table XXXIV. Deer Trypanosomes - Fixed, Giemsa-Stained Culture Forms*

Culture	Age (days)	N	X PK	PN	KN	NA	F	KI	NI	L
N.N.N. 28°										
Deer # 3	10	50	8 (4-14)	7 (3-13)	<1 (0≤1)	6 (4-10)	13 (8-18)	>5	1.1 (0.4-2.0)	26 (16-40)
McCoy's 28°										
Deer # 3	3	50	15 (10-22)	14 (9-21)	<1 (0≤1)	10 (4-15)	13 (7-22)	>5	1.5 (1.0-2.7)	37 (23-48)
Deer # 81	10	100	13 (5-20)	12 (4-20)	<1 (0≤1)	8 (2-13)	12 (5-23)	>5	1.6 (0.5-5.0)	32 (16-48)
McCoy's 37°										
Deer # 3	10	50	8 (2-14)	7 (2-13)	<1 (0≤1)	6 (3-12)	12 (5-22)	>5	1.2 (0.2-3.6)	25 (17-36)

*The abbreviations and format of this table are the same as Table XXXII.

were similar in morphology and size to those from tabanids.

The epimastigotes from deer exhibited the characteristics of T. theileri-like trypanosome culture forms: a pointed posterior end, granular cytoplasmic inclusions, a nucleus near the anterior end or in the middle of the cell (Figure 62), as well as division forms having two nuclei, one anterior to the other (Figure 63).

DISCUSSION

The mammalian trypanosomes are divided into two major sections, the Stercoraria and the Salivaria (Hoare, 1964). Infective forms of stercorarian trypanosomes develop in the posterior portion of the alimentary tract of the vector and their transmission is contaminative, while infective salivarian forms develop in the anterior station and transmission is inoculative. If mammalian trypanosomes evolved from monogenetic trypanosomatid parasites of insects, as most evidence now indicates (Hoare, 1972), the stercorarian mode of transmission (observed in insect trypanosomatid parasites) may be regarded as a more primitive mode of transmission than the salivarian.

In the present study, flagellates were found primarily in the posterior portion of the alimentary tract and never in the salivary glands. Therefore, it may be assumed that these organisms are transmitted by contaminative transmission and not by inoculation of infective material during biting. If most of these tabanid flagellates are stages in the life-cycle of mammalian trypanosomes, their stercorarian mode of transmission indicates a primitive association between vector and parasite.

A close relationship between insect monogenetic trypanosomatid parasites and the vector forms of primitive mammalian trypanosomes is indicated by their identical modes of transmission as well as their morphological similarities.

Hoare (1923) studied the various forms of the stercorarian Trypanosoma (Megatrypanum) melophagium occurring in its sheep-ked vector, Melophagus ovinus. After comparing forms found in adjacent portions of the insect gut, Hoare proposed a developmental scheme to explain the relationships of the forms observed. The sequence of forms occurring in the mid to posterior portions of the intestine were "typical crithidia - transitional crithidia - pyriform crithidia - metacyclic trypanosomes." Hoare's "crithidia" forms are now correctly referred to as epimastigote forms (Hoare and Wallace, 1966). The latter term will be used in the present discussion. Hoare's descriptions and illustrations of the epimastigote and amastigote forms of T. melophagium are identical to the epimastigote and Type I amastigote forms observed in tabanids. The pyriform epimastigotes and amastigotes observed in the hindgut and rectum of both sheep-keds and tabanids are very similar in shape, size and behavior to the Crithidia species of insects. However, the lateral emergence of the flagellum in the epimastigotes and the posterior position of the kinetoplast in the amastigotes of T. melophagium and the tabanid flagellates separate them from Crithidia (choanomastigotes). As discussed earlier (Introduction), the structural detail concerning the position of the kinetoplast was apparently overlooked by Wallace (1962) when he regarded Nöller's "barley corn" forms (1916, 1925) as Crithidia species rather than infective forms of T. theileri. The similarity of pyriform epimastigotes and amastigotes to Crithidia was emphasized by Swingle (1909), who compared them to Léger's

"formes grégariniennes" (Crithidia Léger 1902).

Developmental Sequence of T. theileri-like Trypanosomes Observed in Tabanids

The morphological characteristics and locations of different forms of tabanid flagellates are so similar to those described by Hoare (1923) for T. melophagium that a similar developmental sequence may be proposed for both sets of flagellates. Such a scheme, using the flagellate forms observed in tabanids, is presented in Figure 64. Hoare's observations of T. melophagium will be compared with those of the present study.

Hoare (1923) described the gradual transformation of ingested trypanosomes into metacyclic trypanosomes (metatrypanosomes) as they progress through the alimentary canal of the sheep-ked vector. Since T. melophagium, like the T. theileri-like trypanosomes, is very scarce in the blood of healthy vertebrate hosts (Hoare, 1972), an insect vector must imbibe a very small number of organisms during blood-feeding. The fact that no intermediate stages between trypomastigote and epimastigote forms were observed in sheep-keds or tabanids suggests that epimastigotes in the insect gut arise by division from a few or a single ingested trypanosome.

The midgut of the keds, as in the tabanids, contained typical epimastigotes (cf. Figure 12) while the anterior portion of the hindgut to the rectal junction was densely lined with

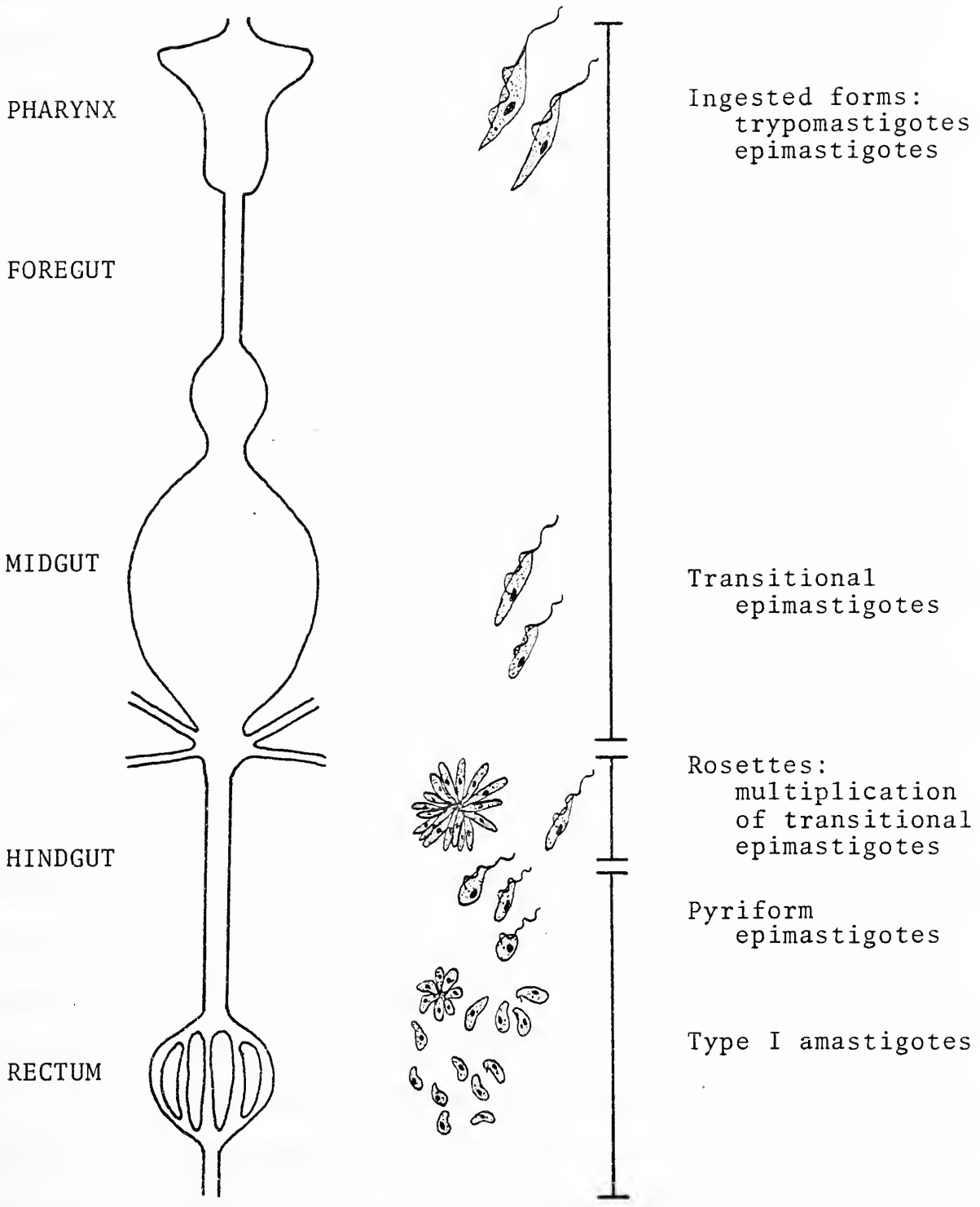


Figure 64. Proposed Developmental Sequence of a T. theileri-like Trypanosome in a Tabanid.

transitional forms (intermediate forms between typical epimastigotes and small metacyclic trypanosomes). Though dividing epimastigotes were frequently observed in the midgut of the ked, few were observed in the tabanid midgut. Hoare described transitional epimastigotes in dividing rosette formations in the iliac bulb (i.e., anterior hindgut) (cf. Figures 15-16). The transitional epimastigotes were regarded as one phase in the transformation of elongate epimastigotes into pyriform epimastigotes. The latter forms were described as transitional epimastigotes which had become swollen and club-shaped. The flagellum and undulating membrane of these forms were reduced. The small pyriform epimastigotes had "either a truncated anterior end and no free flagellum or, pointed anterior end and a small flagellum" (Hoare, 1923). All pyriform epimastigotes had the typical juxtanuclear kinetoplast and frequently the nucleus was near the posterior end of the cell. Dividing forms were sometimes seen. Those pyriform epimastigotes having a flagellum are identical to the short epimastigotes seen in tabanids (Figures 10, 13, 64). Those forms without a free flagellum are analogous in morphology to the Type I amastigotes observed in tabanids (Figures 10, 17, 18, 64).

The most distal portion of the hindgut of the ked exhibited metatrypanosomes which were pyriform cells having posteriorly placed kinetoplasts and flagella which ran along the edges of the cells and, in some cases, emerged as free flagella. Metatrypanosomes were not distinguished from Type I amastigotes in the tabanids examined. Though only a tip of a

flagellum could occasionally be observed emerging from a Type I amastigote, this flagellated form may be analogous to the metatrypanosomes described by Hoare.

As in the tabanids, most of the pyriform and metatrypanosomes (? flagellated Type I amastigotes) occupied the hindgut of the ked and were found less abundantly in the rectum. Hoare regarded the metatrypanosomes as the infective forms of the trypanosome. In addition, he described typical rounded leishmanial (amastigote) forms intermixed with other forms in the hindgut lumen. Hoare (1923) provided some evidence for regarding these forms as "by-products of any given stage." He suggested that they are "produced when the flagellates are overcrowded or when they are degenerating" (Hoare, 1972).

Hoare (1923) regarded the iliac bulb (anterior hindgut) of the ked as the primary site of development and transformation of flagellates. The colon (posterior hindgut) was described "as a canal in which the parasites occur only in transit" and the rectum as a region in which very little further development occurs (Hoare, 1923). These observations apply equally to infected tabanids.

The striking similarities of the vector forms of the sheep Megatrypanum trypanosome to the majority of tabanid flagellates observed in this study support the hypothesis that the tabanid flagellates represent developmental stages in the life cycle of a T. theileri-like (Megatrypanum) trypanosome.

Metacyclic (Infective) Trypanosome Stages in Tabanids

The occurrence of epimastigotes and trypomastigotes in cultures from two tabanid species harboring Type I amastigotes is consistent with the view that these immotile forms are the precursors of a Trypanosoma species.

Amastigote forms resembling the Type I amastigotes in this study have been observed in the posterior intestinal segments of other wild tabanid species. These observations include the following species: Haematopota duttoni and H. vandenbrandeni (Rodhain et al., 1913), H. italica (Léger, 1904), H. pluvialis (Nöller, 1916; Jegen, 1924), Tabanus africanus, T. ditaeniatus, T. fasciatus, T. gratus, T. par, T. socius and T. virgatus (Wenyon, 1908) and T. secedens, T. fuscomarginatus and T. thoracinus (Bruce et al., 1911). Wallace (1962) regarded the immotile forms in these species as Crithidia species. However, Hoare (1972) recognized them as metatrypanosomes and listed the various names by which they were originally described in synonomy with Trypanosoma theileri Laveran, 1902.

Type I amastigotes also closely resemble vector stages of other insect-borne vertebrate Trypanosoma species. Such forms include the haptomonad stages of the rodent trypanosome T. zapi found in its flea vector, Megabothris abantis (Davis, 1952) and analogous stages of the avian trypanosome T. avium from its hippoboscid vector, Ornithomyia avicularia (Baker, 1956).

Crithidia Species in Tabanids

The choanomastigote forms in tabanids and in cultures were clearly distinguishable from the diverse epimastigote forms observed. Therefore, such Crithidia species, as seen in their motile stages, may be easily excluded from a consideration of the vector stages of a mammalian trypanosome. However, the immotile forms of Crithidia are not easily distinguished from resting stages of other genera.

The appearance of Crithidia species # 2 and epimastigote forms in a culture inoculated with Type I and II amastigotes from a single fly (T. quinquevittatus) provided evidence that the two types of immotile forms represent the precursor stages of two distinct genera. The supplantation of epimastigote forms by Crithidia in the culture after 4 weeks was consistent with the pattern of slow growth of a Crithidia species (C. rileyi) described by Wallace (1962). He rejected Nöller's observation (1916) that epimastigotes (? T. theileri) grew from fly forms which Wallace regarded as Crithidia species. He reasoned that, if the Crithidia seen in the tabanid grew slowly in culture, some previously unrecognized fly forms could have grown initially as epimastigote forms and effectively excluded the Crithidia from the culture. Wallace's supposition explains the above situation seen in the present study; however, the tabanid forms observed by Nöller, and those designated Type I amastigotes here, do not conform to the description of the genus Crithidia. Nöller's "barley corn" forms and the Type I

amastigotes are most accurately described as pyriform amastigotes and probably represent the metacyclic (infective) stages of a Trypanosoma theileri-like species.

Vector Forms in Culture

In general, trypanosomatid forms seen in culture at 28° are regarded as vector stages and those which grow at 37° are regarded as vertebrate stages of a trypanosome species (Hoare, 1972). In the present study, 98% of the cultures grown at 28° (Tables XXVII-XXVIII) exhibited epimastigote forms while only 32% of infected flies (Table XXIV) harbored epimastigote forms. The majority of infected tabanids (94%) exhibited amastigote forms.

The infected tabanids found swarming at Site II (Table XVI) represent 32% of the tabanids infected with epimastigote forms caught in 1973. The majority of the infected swarming flies (57%) were blooded. The high incidence of epimastigote forms in recently blooded flies suggests that the epimastigote stage is found soon after the fly becomes infected. However, it is a transient form which is transformed into immotile stages as illustrated in Figure 64.

No transformations of epimastigotes to trypomastigotes occurred when culture forms were transferred from 28° to 37° (Table XXX). This observation is consistent with most reports about the cultivation of T. theileri-like trypanosomes from cattle. Trypanosome cultures grown at 28° generally exhibit

epimastigote forms (Splitter and Soulsby, 1967) and a few trypomastigotes (Herbert, 1961, 1965c). The transfer of 28° cultures to 37° inhibits the growth of epimastigotes of Trypanosoma species (Herbert, 1965c; Deane and Kirchner, 1963) and rarely causes transformation of these forms into trypomastigote stages. Trautmann (1922), as reported by Mesnil (1923), while studying T. theileri from cattle, made the only observation of the transformation of T. theileri-like epimastigotes isolated at 28° into trypomastigotes at 37°.

Trypomastigote forms have occasionally been observed in cultures grown at 37° (Splitter and Soulsby, 1967; Ristic and Trager, 1958). These observations are consistent with the occurrence of trypomastigotes in two initial 37° cultures (CuF_{10}) inoculated with tabanid forms in this study. Splitter and Soulsby (1967) reported that 37° trypomastigote forms could be subcultured at 27° to produce epimastigote forms and these, in turn, could be subcultured at 37° to produce trypomastigote forms. The scarcity of such observations in which culture temperature change is directly associated with morphological transformation indicates that several variables are important. The type of culture medium, the source of the inoculum, and the age of the parasites probably influence the occurrence of different forms seen in cultures at different temperatures.

Immotile forms resembling Type I amastigotes have very infrequently been observed in culture. Wells (1971b) identified pear-shaped forms in cultures of T. theileri-like trypanosomes from cattle. He suggested that such forms are identical

to the "barley corn" forms of Nöller (1925) and thus represent the metacyclic stage of the trypanosome.

Host-Parasite Relationships of Trypanosoma theileri-like Trypanosomes

Trypanosoma theileri, like T. melophagium and other Megatrypanum species, is an example of a parasite which has endured a long association with its host. The latent trypanosome infections and the lack of pathogenicity and sterile immunity in the hosts (Wells, 1972) are characteristic of a well-adapted host-parasite relationship. This close biological association may account for the limited success of immunological tests which rely on the presence of mammalian antibodies to T. theileri-like organisms.

Splitter et al. (1967), using the indirect hemagglutination test, could not demonstrate antibodies to T. theileri when screening infected cattle. However, these investigators found precipitin by agar gel immunodiffusion in 10 out of 17 infected cattle even though this test is approximately 6000 times less sensitive than the hemagglutination method (Davis et al., 1967). In view of the limited results obtained in the past, it is not surprising that no antibodies could be detected in ruminant sera in this study.

Potential Vertebrate Hosts of Tabanid Flagellates

The observation that all tabanids infected with trypanosomes are parous, gravid or blooded, and the evidence suggesting that the most frequently infected (and most abundant) species are anautogenous, indicate a high correlation between infection and blood-feeding. In addition, the seasonal pattern of infection is consistent with the possibility that trypanosomes are multiplying in a vertebrate host during the fly season (Table XII). If tabanids in this study obtained most of their flagellates from vertebrates, potential hosts should be considered.

Schlafer (1973) cultured blood from cattle examined at the New York State College of Veterinary Medicine and isolated T. theileri-like trypanosomes from approximately 44% of the total (158) samples. Some of the blood was obtained from cattle with veterinary problems; therefore, the trypanosome infection rate may be slightly higher than in a random sample of healthy cattle. Glaser (1922) found 25% (of 28) cattle infected in New Jersey, and Hare et al. (1969) found 29% (of 73) of a healthy herd of cattle in Pennsylvania infected. There is little doubt that domestic cattle may serve as a source of T. theileri-like organisms. However, the large number of infected flies collected at sites distant from cattle (Sites II, IV, Table XI) suggests that an alternative source may be present. Such a speculation led to the sampling of white-tailed deer for trypanosomes.

The isolation of T. theileri-like trypanosomes from all of the deer sampled during the summer months (Table XXXIV) indicates that these animals are a potential source of tabanid flagellates. In July, 1973, Tabanus quinquevittatus and T. sulcifrons were observed persistently attacking deer at the New York State Game Farm.

Adaptations for the Transmission of Tabanid Trypanosomes

The transmission of blood-borne parasites by haematophagous vectors is dependent on a combination of adaptations of the host and vector. The parasite must occur in the peripheral blood at a time when the vector is likely to feed on the host. Furthermore, the parasite stage occurring in the blood must be infective to the appropriate vector.

The natural transmission of T. theileri-like trypanosomes has never been studied. However, the experimental transmission studies of Kraneveld (1931) indicate that tabanids have great potential as vectors of these organisms. Fewer studies of T. theileri-like organisms from wild ruminants than from domestic animals have been made. Keymer (1969), during a study of Trypanosoma (Megatrypanum) species of the duiker in Africa, examined tabanids and hippoboscids in search of a possible vector. Dissections of 62 Haematopota specimens, 79 Tabanus specimens and 132 hippoboscids (Echestypus paradoxus) failed to reveal any trypanosomes. Despite these results, Keymer noted the close association of hippoboscids and antelope species known to

be hosts of T. theileri-like trypanosomes and, on this basis, suggested that these flies be seriously considered as possible vectors of the trypanosomes.

One adaptation for the transmission of blood parasites is the occurrence of infective forms in the peripheral blood of the hosts during periods when the vectors are active. This co-adaptation occurs in various forms of filariasis and plasmodial infections (Worms, 1972). Recent evidence indicates that the anuran Trypanosoma rotatorium may also show periodic parasitemias which may be correlated with the availability of its insect vectors (Desser et al., 1973).

The periodic occurrence of organisms in the peripheral blood may also be seasonal. This was discussed earlier (Introduction) in regard to the difficulty of finding T. theileri in the blood of cattle during the winter months. In this study, a culture from one deer (#3) in March failed to exhibit trypanosomes. In August, trypanosomes were readily isolated from the same deer. In tropical areas, T. theileri-like organisms were more frequently reported in blood smears during the rainy season than during the dry season (Wells, 1972). Such seasonal variation may reflect an adaptation for transmission by haematophagous insects.

Blood-borne parasites may be specially adapted to occur in those specific parts of a host which are common vector feeding sites. The abundance of microfilariae of Elaeophora schneideri in the capillaries of the skin on the forehead and face compared to other sections of skin from sheep and elk is regarded

as an adaptation for transmission by its tabanid vectors (Adcock and Hibler, 1969; Hibler et al., 1969). Though no correlations with vector habits were made, Robson and Ashkar (1972) noted higher infections of T. theileri in blood from the ear veins than in blood from the jugular veins of cattle.

The relationship of infective blood forms of T. theileri-like organisms to vector activity was discussed by Wells (1971b). Wells noted a difference in the cultural characteristics of trypanosomes isolated from cattle in the winter from those more frequently isolated in the summer. In the summer, cultured flagellates exhibited longer incubation periods. The number of isolates having increased incubation periods increased as the summer progressed. In conjunction with longer incubation periods, summer isolates retained their infectivity to cattle for longer periods than winter isolates. Wells postulated that peripheral blood forms capable of infecting an insect vector exhibit long incubation periods and long periods of survival. Such characteristics may increase the chances of an infective form being ingested by a suitable vector. Such an hypothesis is consistent with the present study in which the percentage of tabanids infected with trypanosomes increased as the summer progressed.

The information available about host forms of T. theileri-like organisms is compatible with the possibility of natural transmission of these parasites by a tabanid vector.

Trypanosoma (Megatrypanum) Infections in Tabanidae in Central New York State

This study demonstrated that trypanosomatid infections of tabanids occur and are common in central New York State. All of the epimastigote and trypomastigote forms which grew in cultures of tabanid intestinal flagellates were identical to *Trypanosoma theileri*-like trypanosomes from ruminants.

T. theileri-like trypanosomes were cultured from the peripheral blood of cattle and deer which were grazing in areas where tabanids were abundant. The positive correlation observed between blood-feeding and tabanid trypanosomatid infection strongly suggests that the flies obtain the trypanosomes from the blood of these animals.

The tabanid forms of *T. theileri*-like trypanosomes were easily grouped in a developmental pattern which conformed to that described for the hippoboscid vector forms of the closely related species, *T. melophagium*. Therefore, like the latter species, *T. theileri*-like trypanosomes are probably transmitted to their mammalian hosts via the stercorarian route.

Tabanid flies in central New York State harbor monogenetic trypanosomatid parasites of the genus *Crithidia* as well as developmental forms of *Trypanosoma theileri*-like trypanosomes. Therefore, tabanids should be regarded as potential biological vectors of ruminant trypanosomes in central New York State.

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